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# CHRONIC BRONCHITIS[1]

## Its Prevalence, Nature, and Pathogenesis

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In 1939, Dr. Keeton wrote: "The analysis of disease processes rather than of the end results of disease . . . has stabilized and vitalized the medical worker" (1). This declaration of his provides an excellent text for my lecture. It is only by the analysis of various stages in the development of chronic bronchitis that we can hope to find means to prevent and to arrest the progressive disablement that it now inflicts upon so many sufferers, at least in my country.

I suspect that ten years ago members of The American Trudeau Society and Dr. Keeton himself would have been surprised to know that this year you would invite a guest lecturer to speak on chronic bronchitis. For I do not believe this has ever been a very respectable diagnosis in the United States, nor, to judge from your mortality statistics, does the disease appear to be of any great consequence here. A comparison of major causes of death in middle-aged males in England and Wales and in the United States is presented in figure 1. Bronchitis accounts for nearly 10 per cent of the deaths in England and Wales, and the death rate is 45 times greater than yours. It is sometimes suggested that this difference is at least partly an artefact and is due to American doctors being unwilling to certify chronic bronchitis as a cause of death. If this is so, and you really have a disguised mortality from bronchitis, we should find some other equivalent excess under some other heading. The only major excess that you have is of deaths certified as due to coronary arterial disease. I think it is unlikely that a death from bronchitis could be attributed to coronary disease, and we must conclude that there is a much lower mortality from bronchitis and a higher mortality from coronary disease in the United States compared with England and Wales. These differences present a great challenge to medicine and particularly to epidemiologists.

Your morbidity statistics are difficult to interpret and to compare with ours. In their report on sickness experience in selected areas of the United States, Collins and associates (2) listed bronchitis under "Minor Respiratory Diseases," but found it one of the most prevalent conditions, and the "annual disabling case rate per 1,000 persons" was 51. In the recent survey of morbidity statistics from general practitioners in Great Britain (3) the annual "patients consulting rate" for bronchitis was 62 per 1,000 persons. The American bronchitis, however, shows no rise in prevalence in late middle age, and the rate is greater for females than for males, while in England the disease is most prevalent in the elderly and in men. Hence, the word "bronchitis" appears to refer to different things in these two reports. In the recent report on "Chronic Illness in Baltimore" (4), bronchitis was reported in 140 of 11,574 people interviewed at home but, when physicians on the staff of the Johns Hopkins Hospital carried out clinical evaluation of a sample of the population, the diagnosis disappeared without comment. Bronchitis cannot be a consultant's diagnosis in Baltimore. More direct evidence that chronic bronchitis exists in

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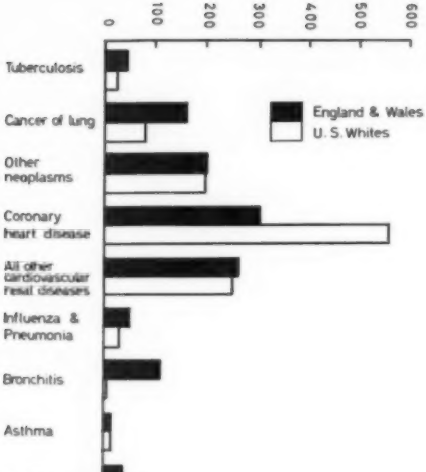


FIG. 1. Death rates of men aged forty-five to sixty-four years per 100,000 from selected causes in the United States and in England and Wales, 1955.

the United States was provided by Pemberton (5) who, in population surveys, made the diagnosis about as frequently as it has been made in surveys in Great Britain (6). However, exact comparison cannot be made because of differences in diagnostic criteria. So far as the evidence goes, it appears that bronchitis exists here in the United States, but that it is probably less disabling in later life and almost certainly less mortal than in Great Britain.

Bronchitis has always been a major problem in Great Britain, and we now have the highest recorded mortality from this disease of any country. The trend of the last thirty-five years of death rates in middle-aged men from major respiratory diseases is shown in figure 2 (7, 8). You will see that the earlier decline in mortality from bronchitis has been arrested, and there has been no fall with the advent of chemotherapy, as there has been in respiratory tuberculosis and pneumonia. It is, of course, possible, when we consider the steeply rising mortality from cancer of the lung, which may be largely attributed to cigarette smoking, that the effects of modern therapy on bronchitis are being masked by an increase in incidence due to cigarette smoking. So far as morbidity is concerned, just over 10 per cent of all episodes of absence from industry because of illness is attributed to bronchitis, which causes a loss of 27 million days work per annum (9).

Although this grave mortality and morbidity have been present for many years, it is only recently that much attention has been paid to chronic bronchitis in our medical literature. I can trace only three articles on the subject between 1935 and 1951. In that year the Association of Physicians of Great Britain and Ireland held a symposium on chronic bronchitis (10). Since 1953 some 10 to 15 articles have been published annually, and three monographs have recently appeared on the subject (11–13). Some of the growing interest in bronchitis in England may be due to the relief from time-consuming concern with tuberculosis by chest physicians since the advent of chemotherapy. Perhaps another reason is that the greatest burden of morbidity and mortality from bronchitis has always been borne by the poorer sections of our community. Mortality from bronchitis is nearly five times as great among unskilled laborers as among the professional and managerial classes (14). Since the introduction of our Health Service, poorer people have had easier access to specialist services, and consultants have been seeing more

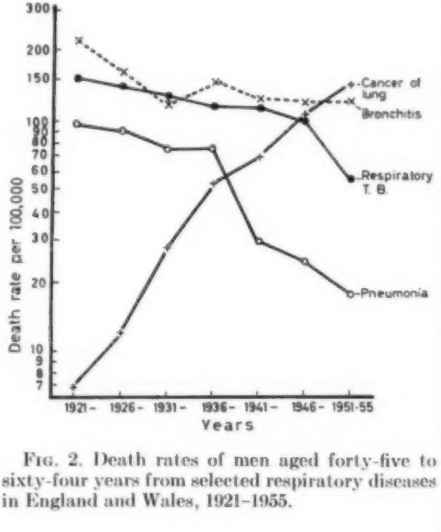


FIG. 2. Death rates of men aged forty-five to sixty-four years from selected respiratory diseases in England and Wales, 1921–1955.

of the diseases that particularly affect such people.

Recent trends in mortality from respiratory diseases in the United States are of interest (figure 3). Since 1950 there has been a steady rise in deaths attributed to emphysema and to chronic interstitial pneumonia, matching the fall in deaths from respiratory tuberculosis. Bronchitis mortality, however, remains low and constant. The explanation for these trends is not obvious, but perhaps they represent an increasing readiness to register deaths as due to emphysema; deaths which, perhaps, might previously have been registered as due to bronchopneumonia or heart disease. Whatever the explanation, if present trends continue, emphysema will be a more frequent cause of death by next year than respiratory tuberculosis.

*The clinical picture of chronic bronchitis:* Before discussing some of the recent studies of chronic bronchitis in Great Britain I must briefly describe the clinical picture upon which British physicians diagnose it.

Three-quarters of the patients are men, mostly over the age of forty. The first essential feature is chronic cough, usually productive of mucoid sputum which may date from childhood but usually comes on first as a "smoker's cough" in middle age. Not infrequently it is said to follow an illness described as acute bronchitis or pneumonia. At first the cough may occur only in winter, with summer remissions; later it may become perennial. In many cases there are recurrent exacerbations of the conditions in which the sputum increases and becomes purulent. These episodes may be described as "chest colds." They are often ignored at first and the patient does not usually seek medical advice until he begins to notice dyspnea. This is usually associated with wheezing and may be described by the doctor as "bronchospasm" because some relief is usually given by bronchodilator drugs. Like the cough, the dyspnea may be of gradual onset but may first be noticed after an acute chest infection. It is almost invariably worse in winter. When it becomes persistent, a clinical diagnosis of "bronchitis and emphysema" is commonly made. In the later stages the patient becomes breathless on the slightest exertion and ultimately dies of respiratory failure, often with cor pulmonale and usually during an exacerbation of bronchial infection. Antibacterial drugs prolong the patient's life, which, for the last five years or more, is often burdensome because of crippling dyspnea.

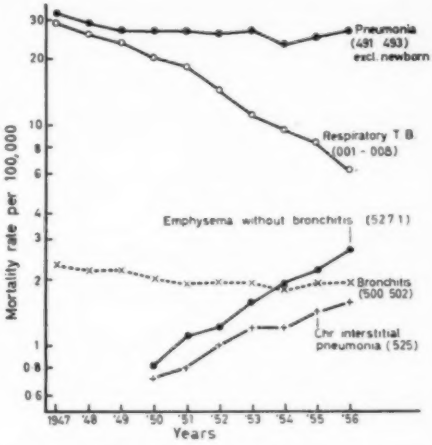


FIG. 3. Crude death rates per 100,000 persons from selected respiratory diseases, United States, 1947–1956.

*Definition and diagnosis:* The clinical picture which I have described is familiar to all British, and I believe to most American, physicians. At any rate, British physicians who come to work in the United States report that they see many of these cases; and yet, as I have shown, the disease is rarely recorded as a cause of death and figures obscurely in your morbidity statistics.

I believe that part of these differences may be due to differences in diagnostic terminology. Clarification of diagnostic terminology is a necessary condition for advancing our mutual understanding of this problem. Indeed, I believe that discrepancies in the definition of diseases and differences in diagnostic criteria are among the most frequent—but least often suspected—causes of discordance between the results of parallel medical investigations. Two investigations into a condition to which the same diagnostic label has been attached may, in fact, be concerned with quite different pathologic states. In other cases the same condition may be investigated under different names. In either case, unavoidable confusion results. This is because we so often fail to define what we are talking about in medicine (15).

In fact, diseases are always difficult to define, partly because of the wide variety in the clinical

manifestation to which any single pathologic change may result in different subjects, but partly because of the inaccuracy and subjective nature of many of the criteria or tests which we have to use in diagnosis and which result in great variation between the results of the same test in different hands. These difficulties are particularly severe in relation to the nonspecific generalized pulmonary disease or diseases of which I have given a broad clinical description, and to which the labels "bronchitis," "asthma," and "emphysema" are rather indiscriminantly attached, either alone or in various combinations. It is time that we attempted to clarify our terminology.

I have been helped in what I have to say on this subject by my participation in a Ciba Guest Symposium held in London last September at which a small group of British investigators managed to reach some provisional agreements on definitions (although what I have to say here represents my own views). I have also been influenced by the results of some recent epidemiologic studies in which I have partaken (16, 17). In relation to the term "chronic bronchitis," I consider that confusion has been caused chiefly by failure to distinguish the essential feature of the disease from its complications. The word bronchitis implies inflammation of the bronchi, not necessarily infection. This inflammation, as in other mucosal surfaces, leads to hypersecretion of mucus, which is manifest in the case of the bronchi as cough with expectoration. In my opinion, the presence of this symptom, in the absence of any cause other than bronchitis, so long as it is persistent or recurrent, is sufficient grounds for the diagnosis of chronic bronchitis. The objection raised against this simple concept of bronchitis (11, 13) is that many subjects who have persistent cough and sputum should not be regarded as having a disease since they are not disabled. Disease, it is argued, only begins when disability from recurrent chest illnesses or breathlessness develops. I maintain, on the other hand, that the inclusion by most authors of breathlessness as an essential symptom to the diagnosis of chronic bronchitis is what leads to confusion, particularly with the diagnosis of asthma and emphysema.

Persistent expectoration is an abnormality of function and merits description as a disease just as much as any other abnormality of function which may not at first cause disability. I might instance dysfunction of the kidney causing albuminuria, or of the pancreas causing hyperglycemia in the absence of symptoms. These are conditions which usually lead to eventual disability. This I believe is commonly true of bronchial hypersecretion which is, in fact, associated with both recurrent chest illnesses and loss of ventilatory capacity.

This is shown by some results obtained in a recent survey of London postmen (16, 17) who were studied by a symptomatic questionnaire and by measurements of sputum volumes and of ventilatory capacity. That chest illnesses causing loss of time from work were most frequent in those producing the larger volumes of sputum is shown in figure 4. In figure 5 it may be seen that, in respect to ventilatory function, while those men who produced less than 2 ml. of sputum during the first hour after rising showed no significant difference from men producing no sputum, only those producing 2 ml. (and most of those producing 6 ml. or more) had reduction in ventilatory capacity. Of course, a prevalence survey such as this cannot reveal the time sequence of productive cough, chest illnesses, and loss of ventilatory capacity. We could only state confidently that bronchial hypersecretion predisposed to these two forms of disability if this had been demonstrated by a prospective survey, but our findings are compatible with this hypothesis. Moreover, the hypothesis fits well with McLean's observations (18) on the action of mucus retention in favoring bronchial infection, and with the evidence that bronchiolitis may play a part in the production of emphysema (19–21).

I suggest that we would gain in understanding and be better equipped to recognize and study bronchitis in its earliest stages before the onset of disability, when preventive action may be effective, if we were to define it in these simple terms: "Chronic bronchitis refers to the condition of subjects with persistent or recurrent excessive mucous secretion in the bronchi." The clinical diagnostic criterion would then be: "Chronic or persistent productive cough, not attributable to localized disease of the lungs, bronchi, or upper respiratory tract." This definition was first suggested by Scadding (10, 15). The exact definition of the words "chronic" or "recurrent" must at present be decided arbitrarily. I would suggest that they should imply that the symptom has been present on most days

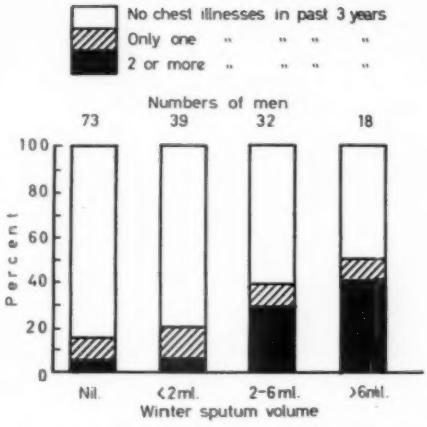


FIG. 4. Frequency of "chest illnesses with an increase of sputum" during previous three years in London postmen aged forty to fifty-nine years, according to volume of morning sputum in winter.

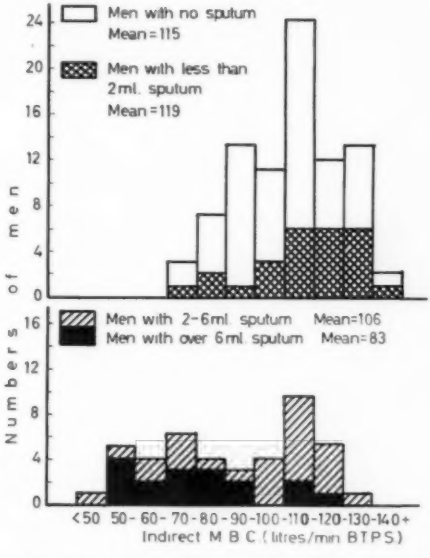


FIG. 5. Frequency distribution of indirect maximal breathing capacity (standardized to age forty and ambient pressure and saturated with water vapor) in London postmen aged forty to fifty-nine according to volume of morning sputum in winter.

for at least three months for at least two successive winters. The severity of the bronchitis thus defined may be graded on the persistence of expectoration throughout the day or, alternatively, on the volume of sputum expectorated in the morning (16).

If this definition of simple chronic bronchitis is accepted, we must consider separately the associated types of disability. The first form of disability is a liability to recurrent chest illnesses. These are difficult to define. Their chief characteristic is an increase or change in the character of the sputum. The difficulty is to decide when such an increase constitutes an "illness." The fact that such an illness causes loss of time from work (23) provides a simple measure, but this criterion is seriously affected by a variety of industrial and economic factors and is impossible to apply to housewives. From the point of view of the pathogenesis of dyspnea, recurrent episodes in which the sputum increases and becomes purulent but without causing serious constitutional effects may be as important as illnesses which appear more dramatic clinically. We need prospective studies to answer this question; meanwhile the exact definition of a "chest illness" cannot be laid down and must vary according to the purpose of any investigation.

The second form of disability, dyspnea, also presents problems of assessment. Clinical assessment is difficult because of the subjective nature of the symptom. In bronchitis it is probably caused chiefly by the greater effort required to produce normal ventilation because of "bronchial obstruction due to reversible spasm, swelling of the mucous membrane, and excessive mucous (24)." If this is accepted, the disorder is best assessed objectively and quantitatively by tests of airway obstruction, of which the most widely used in Great Britain is the volume of air expelled in three-quarters or one second ($F.E.V._{0.75}$ or $F.E.V._{1.0}$) (25). The figures thus obtained are frequently converted to "indirect maximal breathing capacity" (MBC) (as in figure 4) because of their high correlation with the maximal breathing capacity and because clinicians are more conversant with the values thus expressed. There is much to be said for using this test or analogous tests whose results can be related to it because it has already been so widely used. The results may be expressed absolutely when comparing subjects of the same age, or we can correct for age differences (again

TABLE 1
SUGGESTED CLASSIFICATION OF CHRONIC BRONCHITIS

| |
|---|
| **Simple Chronic Bronchitis** |
| Mucoid sputum: |
| Part day (<2 ml. on rising) |
| All day (>2 ml. on rising) |
| **Chronic Bronchitis with Infection** |
| Mucopurulent sputum |
| Recurrent "chest illnesses" |
| **Chronic Bronchitis with Obstructive Lung Disease** |
| Severity: |
| Ventilatory impairment—no dyspnea |
| Ventilatory insufficiency—dyspnea |
| Ventilatory failure—hypoxia and hypercarbia |
| Type: |
| Reversible obstruction ("asthma") |
| Irreversible obstruction |
| Without emphysema |
| With emphysema |

as in figure 4), since the decline of test results with age is approximately linear.

I suggest a classification of chronic bronchitis as in table 1. First, there is simple chronic bronchitis divided into two degrees of severity (16); second, we have chronic bronchitis with infection, causing purulent sputum which may be persistent or recurrent and which may result in clinical exacerbations or "chest illnesses." Third, there is chronic bronchitis with obstructive lung disease. From the point of view of severity, we can subdivide these patients into those with reduced ventilatory capacity but without dyspnea (chronic bronchitis with ventilatory impairment), those with reduced ventilatory capacity and dyspnea (chronic bronchitis with ventilatory insufficiency), and those who also have abnormalities of arterial blood gases (chronic bronchitis with ventilatory failure).

The proper use of the word "asthma" presents difficulties. At the Ciba Guest Symposium we decided that it was best to describe as having asthma those subjects in whom obstruction to the airway changes its severity over short periods of time, either spontaneously or in response to treatment; but we do not yet know enough about the usefulness of this criterion for distinguishing groups of cases of bronchitis with and without asthma thus defined. Nor do we yet know enough about the functional and anatomic pathology of cases of irreversible obstruction to be sure how to diagnose and classify them. Irreversible obstruction is usually regarded as indicating the presence of emphysema. At the Ciba Symposium we concluded that emphysema should be regarded as a morbid anatomic term and that it should not be used as a clinical diagnosis unless the clinical, roentgenographic, and physiologic evidence is such that the presence of the morbid anatomic changes of emphysema can be confidently asserted to be present. I believe that in our present state of knowledge this is often impossible. I have recently been most impressed by cases which showed the clinical and functional abnormalities usually attributed to severe emphysema, but in which at post-mortem examination the anatomic changes of emphysema were extremely mild or narrowly localized. We need much more careful correlation between clinical, functional, and pathologic observations before we can learn how to recognize anatomic emphysema with confidence during life.

Meanwhile, I advocate that we should use the term "chronic bronchitis" in the sense that I have suggested, and that we should make separate reference to associated functional changes in direct descriptive terms rather than in diagnostic terms such as asthma and emphysema, which may mean quite different things to different people. The term chronic obstructive disease of the lungs is a useful one to describe those cases with definite evidence of such obstruction; but it should not be used, as is sometimes done, as a loose description for any chronic breathless subject with a barrel chest.

*Pathology and pathogenesis:* Having attempted to define what I mean by chronic bronchitis, I turn to consider what we know about its pathology and pathogenesis. In fact we know very little about the pathology of simple chronic bronchitis, partly because it has not yet been recognized as a disease meriting pathologic study. Most pathologic studies have been made on patients disabled by recurrent infections or dyspnea and often on those with terminal acute infections. In 1932 Florey and associates (26) described an increase in goblet cells in the bronchi of patients with chronic bronchitis. More recently Lynne Reid (21) has again drawn attention to this overactivity and hypertrophy of mucous glands and goblet cells in chronic bronchitis and has related it to the symptom of productive cough. Dilated openings of mucous glands may sometimes be seen at bronchoscopy and in bronchograms (27). Squamous metaplasia

of the epithelium, infiltration of the submucosa with inflammatory cells, and postinflammatory obliteration of bronchioles are also characteristic features in pathologic descriptions of chronic bronchitis; but whether they are essential features of simple chronic bronchitis or only occur in cases with recurrent infection is not established. Spain and Kaufman (20), McLean (19) and Leopold and Gough (22) have presented evidence that extension of inflammation into the bronchioles is the essential change leading to the development of centrilobular emphysema, and it may well be that it is this extension that results in the development of dyspnea with ventilatory insufficiency in chronic bronchitis.

The only thing we know for certain about the pathogenesis of chronic bronchitis is that it is complex and that many different factors are involved. We would expect that inhaled irritants of various kinds would be the chief cause of simple bronchial hypersecretion, while a variety of intrinsic and extrinsic factors might affect the incidence of subsequent infection and disability. Although mortality figures are based on the last stages of the disease and cannot tell us much about the original causes of bronchitis, they are readily available and provide information which at least poses challenging questions and against which we can test any hypothesis we may make concerning pathogenesis.

There are three main factors that we can learn from British bronchitis mortality figures. The first striking fact is that the male:female sex ratio is about 4:1. We find a similar ratio in our clinical series. I shall return in a moment to consider possible explanations for this. It is interesting (figure 6) to observe that in the very low mortality figures in the United States and Canada the sex ratio is similar to that in England and Wales. The second important fact is the steep gradient of mortality with declining social class to which I have already referred. This is important because it indicates that environment is a major determinant in mortality and, therefore, that preventable agencies are at work. The gradient is not occupational because it is the same in both sexes. We must admit that we cannot explain it. We can suggest overcrowding, atmospheric pollution (for the less privileged classes live in the least salubrious parts of our cities), perhaps less adequate medical care, and economic pressure to continue at work during illness. Overcrowding does not receive any support as an etiologic agent from the investigations of Fairbairn and Reid (28) who found an insignificant correlation between bronchitis mortality and overcrowding.

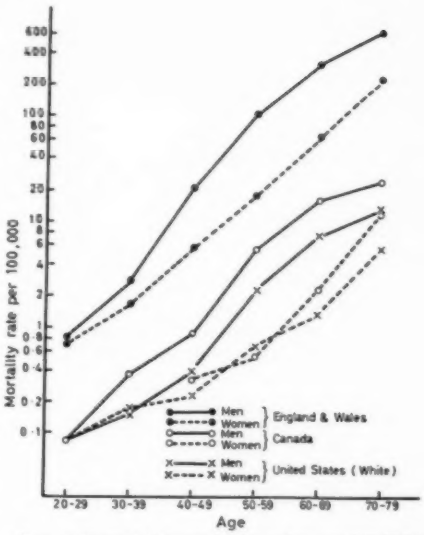


FIG. 6. Bronchitis mortality rates per 100,000 in England and Wales, Canada, and the United States, 1955.

The third pointer from mortality figures is the gradient between urban and rural areas and the significant correlation with indices of air pollution that Fairbairn and Reid and others (29–31) have found. Although the gradient is definite, the standardized mortality in rural areas is only reduced to one half of that in the whole country so that other factors than air pollution must be responsible for the high British mortality from bronchitis.

In recent years a number of population surveys have been carried out in Great Britain, especially by the Pneumoconiosis Research Unit of the Medical Research Council, using standardized symptomatic questionnaires and measurements of ventilatory capacity. It has been found that the prevalence of cough and sputum is the same in rural and urban areas (32, 33) so that urban air pollution does not seem to be an important cause of what I have described as simple bronchitis. The difference between rural and urban areas seems to lie more in a higher frequency of chest illnesses complicating simple bronchitis and a reduction in mean maximal

breathing capacity in the latter. Reid (29) has also shown the special effect of air pollution on recurrent bronchitis in postmen exposed to urban air at their work in contrast to clerks working indoors.

Second, an increased prevalence of disabling bronchitis has been found among coal miners, in whom it bears some relation to duration of dust exposure (34). Third, and perhaps most important of all, there is the effect of tobacco smoking. The close correlation between bronchitis and tobacco smoking has been shown in many recent investigations. The disease is, in fact, seldom seen in men who have never smoked. The observations of Higgins (23) in random studies of men from fifty-five to sixty-four years of age in four communities in Great Britain are set forth in figure 7. The striking effect of present smoking habits on cough and sputum (simple bronchitis) is shown, and the association with chest illnesses and dyspnea, which, however, are not so closely related to present tobacco consumption. Browne and co-workers (35), in a survey of elderly men carried out in general practices in Birmingham, found that the social-class gradient in the prevalence of bronchitis only appeared in smokers. This interesting observation suggests that in the causation of disabling bronchitis the effect of smoking requires potentiation by some other factor correlated with social class.

It has been suggested by Oswald and Medvei (36) that differences in male and female smoking habits may account for the different sex incidence of the disease, for they found a similar prevalence of bronchitis among civil servants of both sexes with similar smoking habits. I do not think this is the whole story. Although in our recent survey of male and female post-office workers (16) we found a similar prevalence of cough and sputum in heavy smokers of both sexes, it appeared that the secondary effects of cough and sputum were less marked in women. In the women, unlike the men, there was no significant depression of ventilatory capacity in association with productive cough. The numbers of subjects were too small for us to be confident of the significance of this finding, but I believe that women are for some reason or other less susceptible to the disabling consequence of bronchial hypersecretion than are men.

It is certainly clear that not all smokers, or urban residents, develop bronchitis, so that individual susceptibility must be important in its pathogenesis. There is some evidence of familial susceptibility (12, 37). Mulder (38) considers that there may be an allergic basis for this, partly because of the frequency with which he finds eosinophils in the sputum. We do not observe eosinophils so frequently and note them chiefly in subjects with reversible obstruction (39). An increased prevalence of allergic disorders in the families of subjects with bronchitis as compared with controls was found by Ogilvie and Newell (12). We need more information about this question.

Summarizing my views of the pathogenesis of chronic bronchitis in the light of these and other observations, I suggest that atmospheric irritants (chiefly cigarette smoke) produce bronchial hypersecretion in susceptible subjects. In some of these, especially in the poorer sections of the population exposed to atmospheric pollution or

Fig. 7. Prevalence of respiratory symptoms and "chronic bronchitis" (defined as cough and sputum with at least one chest illness in past three years) in samples of men aged fifty-five to sixty-four years in four communities in Great Britain (Higgins (23)).

dusty working environments, secondary recurrent or persistent infection develops and leads to disability from bronchial obstruction and ultimately to pulmonary emphysema. This hypothesis is reasonable and fits most of the facts.

Two important facts, however, remain unexplained. The first is the reason for a steep social-class gradient in morbidity and mortality. We have not yet identified the factors responsible for this. The other unexplained fact is the excessively high mortality in Great Britain compared with other countries and especially with the United States. We do not yet know whether the prevalence of simple bronchitis is much higher in Great Britain than elsewhere. Olsen and Gilson (40) found a lower prevalence of respiratory symptoms in Bornholm than in rural Britain, which they ascribe in particular to the smaller proportion of cigarette smokers in Bornholm. Until comparative surveys using standardized techniques have been made in this country, we cannot say what sort of prevalence you have here. Pemberton's surveys (5) in New England suggest that the prevalence of simple bronchitis may not be greatly different in Great Britain and in the United States. If this is so, we would have to explain why we have such a higher prevalence of disabling and fatal bronchitis.

I am tempted to speculate that one peculiar habit of the British may play some part in this. I think we are the only people in the world who normally sleep in unheated bedrooms with the windows wide open. Wright and Wright (41) found a very high correlation between temperature and mortality in infants and the elderly from bronchopneumonia in London. I wonder whether the chilly bedrooms which the British enjoy (or endure) may reduce the resistance of the hypersecreting bronchus to infection so that simple bronchitis becomes more frequently complicated by disabling infection in Great Britain than anywhere else in the world.

* * *

I believe that the problems that I have presented to you today would have interested Dr. Keeton, particularly with respect to the effects of environment upon the etiology of this disease. I hope I may have aroused your interest, too. If we are to reduce the wastage and suffering that chronic bronchitis causes in Great Britain, we need not only extend our own epidemiologic studies; but we also need comparative studies in other countries where the disease appears to run a different course. We must improve our diagnostic methods so that we may learn to distinguish different forms and manifestations of the disease. If what I have said encourages any of you to enter this field of research and thus to increase our understanding, I shall have even greater cause for gratitude to you than that which I already have because of the honor you have paid me and the pleasure you have given me by inviting me to take part in your meeting here in Chicago.

## Summary

Mortality from bronchitis in England and Wales is about forty-five times greater than in the United States. Although some of this excess mortality may be due to differences in diagnostic custom, much of it must be real. No strictly comparable morbidity statistics are available, but there is some evidence that the disease may be common in a less disabling form than in Great Britain.

Clarification of diagnostic terminology is necessary if the reasons for these differences are to be elucidated. At present there is much confusion over the use of the words "bronchitis," "asthma," and "emphysema."

It is suggested that chronic bronchitis should imply no more than hypersecretion of bronchial mucus causing persistent or recurrent productive cough. Evidence is presented that such hypersecretion is significantly associated with an increased liability to recurrent bronchial infection and reduction of ventilatory capacity. These associated forms of disability should be regarded as complications of simple bronchitis and should be described and classified separately.

The proper use of the word asthma, indicating reversible bronchial obstruction in association with bronchitis, requires further clarification. Emphysema should be regarded as a morbid anatomic term and should be used only to describe cases in which there is clear evidence of its presence.

The essential pathology of chronic bronchitis is probably hyperplasia and hypertrophy of mucus-secreting glands. Secondary infection is probably responsible for irreversible obstruction and emphysema.

Inhaled irritants, including polluted air and dust—but particularly tobacco smoke—are concerned in the pathogenesis. The factors responsible for high British mortality and the

steep social-class gradient have not yet been identified. More epidemiologic and clinical studies extending to other countries are needed.


## Sumario

*La Bronquitis Crónica. Su Frecuencia, Natrualeza y Patogenia*

La mortalidad debida a la bronquitis en Inglaterra y Gales es unas cuarenta y cinco veces mayor que en los Estados Unidos. Aunque alguna parte de esta mortalidad excesiva quizás se deba a diferencias er los hábitos de diagnóstico, una gran parte debe ser real. No hay a mano estadísticas de morbidad absolutamente comparables, pero existen algunas pruebas de que la enfermedad tal vez sea común en forma menos incapacitante que en la Gran Bretaña.

El esclarecimiento de la terminología de diagnóstico resulta necesario si van a dilucidarse las razones de esas diferencias. Actualmente reina mucha confusión en cuanto al empleo de los vocablos bronquitis, asma y enfisema.

Sugiérese que el término de bronquitis crónica no debería denotar más que hipersecreción de mucosidades bronquiales, ocasionando tos húmeda persistente o recurrente. Ofrécense pruebas de que dicha hipersecreción se vincula significativamente con una propensión mayor a infección bronquial recurrente y disminución de la capacidad ventiladora. Hay que considerar estas formas asociadas de incapacidad como complicaciones de la bronquitis simple, describiéndolas y clasificándolas por separado.

El empleo debido del vocablo asma, indicando oclusión bronquial reversible unida a bronquitis, necesita mayor esclarecimiento. El enfisema debe ser considerado como término anatomopatológico y no debe usarse el término más que para describir casos en que existen signos manifiestos de su presencia.

La patología esencial de la bronquitis crónica es probablemente hiperplasia e hipertrofia de las glándulas mucosecretorias. La infección secundaria es probablemente la causa de la oclusión irreversible y del enfisema.

Los irritantes inhalados, comprendiendo aire contaminado y polvo—pero en particular el humo del taboco—figuran en la patogenia. No se han identificado todavía loss factores causantes de la elevada mortalidad en Inglaterra y de la empinada pendiente que separa allí las clases sociales. Se necesitan más estudios epidemiológicos y clínicos que abarquen otros países.

## Resume

*Bronchite chronique: Prédominance, Nature et Pathogénie*

La mortalité par bronchite en Angleterre et au pays de Galles est environ quarante-cinque fois plus élevée qu'aux États-Unis. Bien que cet excès de mortalité puisse être attribué aux différences dans les habitudes diagnostiques, il est probablement en grande partie réel. On ne dispose pas de statistiques de morbidité strictement comparables, toutefois certaines données indiquent que cette affection peut être commune, mais sous une forme moins invalidante qu'en Grande-Bretagne.

Une clarification de la terminologie diagnostique est nécessaire si l'on veut élucider les raisons de ces différences. Il règne actuellement une grande confusion quant à l'usage des mots bronchite, asthme et emphysème.

L'auteur est d'avis que le terme bronchite chronique ne doit impliquer rien de plus qu'une hypersécrétion de mucus bronchique entraînant une toux persistante ou récurrente avec expectoration. Il fournit la preuve qu'une telle hypersécretion est nettement associée à l'accroissement du risque d'infection bronchique et à la réduction de la capacité de ventilation. Ces formes d'invalidité associées doivent être considérées comme des complications de la bronchite simple et décrites séparément.

L'emploi approprié du mot asthme, indiquant l'obstruction bronchique réversible associée à la bronchite demande à être plus amplement clarifié. L'emphysème doit être considéré comme un terme d'anatomie pathologique qui ne serait utilisé que pour décrire les cas où sa présence est nettement démontrée.

L'anatomie pathologique essentielle de la bronchite chronique est probablement l'hyperplasie et l'hypertrophie des glandes sécrétrices de la muqueuse bronchique; l'infection secondaire est probablement responsable de l'obstruction irréversible et de l'emphysème.

Les agents irritants, y compris l'air pollué et la poussière—en particulier la fumée de tabac—sont intéressés dans la pathogénèse. Les facteurs responsables de la haute mortalité en Angleterre et le gradient élevé de la classe sociale n'ont pas encore été identifiés. De plus amples recherches épidémiologiques et cliniques s'étendant à d'autres pays sont nécessaires.


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# VACCINATION AGAINST TUBERCULOSIS WITH NONLIVING VACCINES

## I. The Problem and Its Historical Background

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REVIEW OF LITERATURE—*Continued*

### 1926–1930

In a limited experiment, Boquet, Nègre, and Valtis (1926) vaccinated a few guinea pigs with filtrates of the peritoneal exudates of tuberculous animals which had received an intraperitoneal injection of tubercle bacilli (113). The filtrates were reported to protect normal animals against a subsequent light subcutaneous injection with bovine tubercle bacilli, but it has been pointed out by Valtis (1926) that these filtrates probably still contained living tubercle bacilli (114).

Dienes and Schoenheit (1926) exhaustively extracted dead tubercle bacilli with ether, alcohol, and chloroform, and vaccinated groups of 6 to 12 guinea pigs with various doses by repeated intracardial, intradermal, or subcutaneous injections (115). The animals were infected five to ten weeks after vaccination by the intracardial or subcutaneous route, and sacrificed thirty-three to sixty days later to compare their extent of tuberculous disease with that of nonvaccinated control animals. No evidence of immunity was found, and the writers reported that the nonvaccinated controls actually appeared to fare better. It must be pointed out, however, that other workers have reported that exhaustive extraction with organic solvents removes a part, or all, of the immunogenic moiety of tubercle bacilli (108, 241).

In line with Besredka's concepts of organ-specific immunity (116), Jizuka (1926) introduced heat-killed tubercle bacilli ("Besredka's antigen") into a few guinea pigs by the intratracheal route, and compared the extent of pulmonary tuberculosis after challenge infection by the same route with that of nonvaccinated animals (117). Jizuka concluded that "the comparison of lesions in the two categories of animals indicates that the lungs of guinea pigs treated by the tracheal route were more resistant to the tuberculosis organism than those of normal guinea pigs."[9]

In an extensive discussion of immunization against tuberculosis, Long (1926) emphasized the historically based prejudice in favor of living vaccines against tuberculosis:

> The success of Pasteur with attenuated anthrax and chicken cholera germs had prepared the way, the example of Jenner was a constant stimulus. . . . It is significant that among the very first studies were experiments on the immunizing properties of *living* tubercle bacilli. Immunity following infection was an unescapable clinical fact in the case of other diseases, and . . . . this more dangerous method of immunizing appeared to many early investigators more likely to be effective than the use of specific material made harmless by heat, chemicals or other reason" (italics added) (118).

Long also pointed out the artificiality of refusing the term "immunity" to acquired resistance against tuberculosis. Granting that the degree of immunity following vaccination with cowpox virus, or seen in survivors of typhoid fever, was not bestowed by contact with the tubercle bacillus, Long held that the greatly enhanced resistance which does develop must be considered as "nothing less than immunity to infecting doses below a certain size." Although his review was largely limited to a consideration of experiments with living tubercle bacilli and did not cover the literature of vaccination with nonliving vaccines, Long nevertheless concluded that hope for an effective antituberculosis vaccine lay in living preparations.

[9] "La comparaison des lésions dans les deux catégories d'animaux indique que, chez les cobayes préparés par la voie tracheale, les poumons resistent mieux au virus tuberculeux que chez les cobayes neufs."

Convincing evidence for the efficacy of nonliving vaccines was brought forward in the years 1926 to 1930 by Petroff and his co-workers (119–122). Repeated experiments with different vaccines were conducted in guinea pigs, employing statistically significant numbers of animals in the various experimental and control groups. The efficacy of vaccines was measured by the survival time of vaccinated animals after challenge, and also by the extent of tuberculous disease in randomly selected animals sacrificed at intervals after the experimental infection. The mean survival time of animals vaccinated by three intraperitoneal injections of 2.5 mg. (dry weight) each of heat-killed human tubercle bacilli at fourteen-day intervals was found to be 80 per cent longer than that of control animals. The extent of disease of the vaccinated animals sacrificed at different times after subcutaneously administered challenge infection was considerably less than that of the controls. In contrast, groups of animals vaccinated with Dreyer's "diaplyte" (82) showed only a minimal degree of protection (119), thus again illustrating the importance of the methods of killing and preparing nonliving vaccines.

The immunity bestowed by the heat-killed preparations was generally of the same order as that elicited by large quantities of living BCG in comparable experiments (122), and was evident regardless of whether the challenge infection was relatively light or quite heavy (250 to 44,000 virulent bacilli) (121, 122). When challenge infection was made by the intratesticular route, employing only 50 virulent tubercle bacilli, the animals vaccinated with killed vaccine outlived those given living BCG by a considerable margin (a mean survival time of 401 versus 267 days, as compared with 229 days for a control group) (122). When vaccination was accomplished by repeated feedings of the vaccines and challenge infection by inhalation of a cloud of virulent tubercle bacilli, the heat-killed preparation afforded some protection, and living BCG, none (122). Killed vaccines were also effective in prolonging the life of intracardially infected animals (121). Not all of the animals showing heightened resistance to challenge infection were sensitized to tuberculin by the vaccination (122).

Petroff thought it very questionable that a vaccine of any type capable of eliciting complete protection against tuberculosis could be developed, and stressed the clinical value of vaccines capable of only a quantitative increase in resistance. In reviewing the literature of vaccination against tuberculosis, he found that "perhaps the bulk of experiments since the discovery of the tubercle bacillus have accumulated in the attempt to establish immunity with dead organisms" (120), and concluded that the great majority of these studies indicated that "the animals sensitized with dead tubercle bacilli do not vary from those infected, anatomically, allergically, or immunologically" (120). Petroff underlined the importance of vaccinating with adequate doses: "It is true that in order to produce the same intensity of reaction many times more dead tubercle bacilli than living organisms must be used, but it must not be forgotten that dead material does not propagate when injected into the body" (120). Pointing out that not a single one of his experiments, regardless of variation in methods and materials, failed to show some immunologic activity by killed vaccines, he concluded that the failure of others to demonstrate their efficacy was probably due to such experimental artifacts as the employment of suboptimal quantities of vaccine, the unnatural severity of challenge infection and, frequently, the extraneous loss of animals from intercurrent infection.

Lange, Freund, and Jochimsen (1927) again expressed the view that many nonliving vaccines had been evaluated as ineffective only because it was hoped that a preparation giving total immunity would be found (123). They believed that, with the development of a more realistic attitude toward tuberculosis prevention, the expectations made of a vaccine would become more modest and that preparations capable of retarding, though not preventing, a later tuberculous infection would be rightly adjudged as valuable. In a few limited experiments, they found some evidence of resistance to disease in guinea pigs vaccinated with heat-killed tubercle bacilli and challenged by the intradermal route.

Lange, Jochimsen, and Magat (1927) conducted vaccination experiments with heat-killed bovine tubercle bacilli, and with "Schröder's vaccine" in rabbits (124).

The Schröder preparation was made by incubating tubercle bacilli for several weeks in spleen and thymus extracts on the supposition that these organs play an important role in immunity to tuberculosis. Schröder (1927) found that tubercle bacilli so exposed were no longer infective for

guinea pigs and possessed a "high degree" of immunogenic as well as therapeutic activity (125). Lange's group found the heat-killed preparation to be more effective than Schröder's vaccine. A slight degree of "nonspecific" protection was also elicited in rabbits by injection of "Helpin," a glycerol emulsion of lecithin. The killed tubercle bacilli were more effective in bestowing a degree of acquired resistance when they were administered by the subcutaneous or intradermal than by the intravenous route. Lange concluded that killed vaccines could not prevent, but could significantly slow, the progress of experimental tuberculosis. However, on the basis of a later comparison of their efficacy with that of living BCG he considered the latter to hold more promise (126).

The growing belief that a vaccine capable of reducing the severity of tuberculous disease, although incapable of completely preventing its occurrence, should be considered useful was also voiced by Seiffert (127) who called attention to the fact that the vaccines employed in man against typhus and cholera also fell short of initial expectations (127). In his own experiments, Seiffert sought to "open up" the tubercle bacillus, so to expose its immunogenic antigens. He treated the bacilli with sodium hydroxide and acetone at 45°C., and vaccinated a number of experimental animals and several children with the preparation after removing the acetone and alkali. Subcutaneous vaccination produced no undesirable local effects and was effective in prolonging the life of animals after tuberculous infection.

Zadek and Meyer (1927) reported a follow-up study of 26 children, 17 of them infants, who had received four intradermal injections of Langer's heat-killed vaccine "147" (128). All 26 children were heavily exposed to tuberculous infection at home, but seven were excluded from consideration in this report because of their removal from the source of infection sometime after vaccination. The remaining 19 were free of disease six months to two years after vaccination despite a degree of continued exposure which, in nonvaccinated children, resulted in a very high mortality (the writers cited figures of 25 to 36 per cent).

Löwenstein (1928) vaccinated guinea pigs with tubercle bacilli killed by exposure to sunlight for one year or which had died spontaneously after incubation for four years in a glycerin-bouillon culture (129). Some of the animals which were vaccinated by the subcutaneous or intraperitoneal route did not develop tuberculosis after experimental infection, while others showed a considerably prolonged survival. Whereas Löwenstein nevertheless inclined to the view that living vaccines held more promise, he conceded that nonliving vaccines had not yet been tested sufficiently to permit a rational comparison.

Torikata and Imamaki (1928) believed the immunogenically active substance of tubercle bacilli to be present in a soluble fraction (130). They were able to prolong the life of guinea pigs after direct pulmonary introduction of living tubercle bacilli by previous intrapleural vaccination with "koktoimmunogen," a boiled culture filtrate of tubercle bacilli. The boiled filtrates were less toxic and more effective than similar but unheated preparations.

Arloing and Thévenot (1929) found filtrates of cultures of tubercle bacilli or tuberculous sputum to bestow a degree of protection on guinea pigs against tuberculous infection some months later (131). Arloing, Thévenot, and Josserand (1928) also vaccinated guinea pigs with a mixture of "pulmonary juices" and tapioca, in the hope of stimulating the formation of "lipolytic antibodies," and reported that the process of tuberculous disease after infection was less rapid in these animals than in nonvaccinated ones (132).

In discussing previous attempts at antituberculosis immunization, Gasul (1929) cited Koch as declaring that immunity to tuberculosis could result only from a tuberculous infection, but made no reference to Koch's demonstration of the protective activity of nonliving preparations (133); a similarly misleading reference to Koch's experiments has already been discussed (111). However, Gasul urged further investigation of Langer's heat-killed vaccine.

A comparison of living BCG with several killed preparations in guinea pigs led Kirchner and Newton (1929) to conclude that only the living vaccine could bestow an appreciable degree of resistance against tuberculous infection (134). Although large numbers of animals were employed in this study, its significance is doubtful in view of the high mortality rate due to an intercurrent infection by a pasteurella organism which, in the guinea pig, produces lesions closely resembling those of visceral tuberculosis. Moreover, challenge infection was made by the intradermal route, and the greater resistance of the

BCG-vaccinated animals, if genuine, may have been due, at least partly, to a more pronounced development of a Koch-phenomenon reaction at the site of infection and a resulting expulsion of some of the challenge bacilli.

Kirchner and Schnieder (1929) also found living BCG to be superior to Schröder's killed preparation (125) in a small number of Abyssinian monkeys infected intradermally ten weeks after vaccination by the subcutaneous route (135). However, the investigators expressed the opinion that a greater degree of immunity could be expected from a killed vaccine if a smaller challenge dose were employed. Another, very obvious reason for the superiority of the living vaccine in these experiments is the fact that much greater quantities of living than of nonliving vaccine were given to the respective animals.

E. C. Schroeder and Crawford (1929) vaccinated groups of 50 guinea pigs each with approximately equal quantities (10 to 12 mg.) of living BCG and of a killed bacillary preparation by the intraperitoneal route (136). Five weeks later, challenge infection was begun by introducing virulent bovine tubercle bacilli into the drinking water; this technique was repeated twenty-two times over an eight-week period. Animals vaccinated with either vaccine survived longer than a control group, but the BCG-vaccinated ones exhibited a greater degree of protection. Valtis and Saenz (1929) were unable to bestow "survival protection" on guinea pigs by repeated vaccinations with filtrates of a culture of virulent bovine tubercle bacilli seventy days before direct infection of the eye (137). The lesions of the vaccinated animals appeared to progress somewhat more slowly, however. It is doubtful that the culture filtrates employed as a vaccine were free of living tubercle bacilli (137, 138).

In 1930, Brachman advocated a trial of Langer's vaccine in the United States and reported his employment of the preparation in 5 children exposed to tuberculous infection from parents with open pulmonary lesions (139).

Branch and Cuff (1930) vaccinated guinea pigs with 3-mg. quantities of heat-killed virulent human tubercle bacilli by a variety of routes (140). The animals were infected by intracardial or intracutaneous injection of virulent bacilli twenty-four to forty-two days later. Those vaccinated by the intramuscular, intratracheal, and intraperitoneal routes clearly showed prolonged post-infection survival; those vaccinated by the intramuscular route failed to develop tuberculin skin hypersensitivity as a result of the vaccination. Sabin, Doan, and Forkner (1930) were able to prolong the postinfection survival time of rabbits by vaccinating them intravenously prior to infection with large quantities of certain fractions of tuberculo-phosphatide (141).

A seldom cited, but well-designed and convincing study of nonliving antituberculosis vaccines was reported by Streng and Ryti in 1930 (142). Guinea pigs and rabbits were vaccinated by three repeated injections of tubercle bacilli of a mildly virulent strain killed by heating at 65° C. for thirty minutes. Control groups of animals received a living vaccine or heat-killed *E. coli*. In all, more than 100 guinea pigs and 11 rabbits were used in the experiments. Groups of animals were challenged at different intervals after infection. When challenge took place less than three weeks after vaccination, the course of the disease was similar in vaccinated and control animals. Evidence of an acquired immunity was seen only when at least twenty-five days had elapsed between vaccination and infection, the optimal interval being ninety to one hundred and seventy days. Then the vaccinated animals showed a threefold or greater increase in median survival time over the controls, and the authors stated that "we did not obtain greater success with living than with killed bacilli."[10] When the heat-killed bacilli were coated at 37° C. with beef serum and complement, their immunogenicity was enhanced. Heat-killed *E. coli* was ineffective. Streng and Ryti concluded that acquired immunity against tuberculosis was an impossibility only if immunity were defined as complete and absolute protection, and that the "axiom" that only tuberculous disease could bestow a degree of heightened resistance was not born out by experimental observation. They suggested that the variation in the results obtained by different workers in tuberculosis immunization experiments might be the result of differences in vaccination-challenge intervals.

## 1931–1935

In a brief review of the history of attempts to vaccinate against tuberculosis with nonliving vaccines, Westenrijk (1931) also referred to

[10] "Mit lebenden Bazillen haben wir nicht bessere Erfolge als mit abgetöteten erzielt."

Koch's experiments in this direction as failures, because Koch's killed bacilli were not readily assimilated and induced the formation of local abscesses (143). Westenrijk's thinking here is typical of opinions held by many other investigators, that the induction of local or systemic toxicity by a crude preparation automatically condemns it as a vaccine. (As will be pointed out later, however (*see* 236, 237), the toxigenic and immunogenic properties of killed tubercle bacilli appear to be distinct entities, separable by simple laboratory procedures.) Westenrijk vaccinated rabbits by several routes with heat-killed bacillary preparations (Besredka's antigen) and infected them four and one-half months later. The vaccinated animals lived longer and had less tuberculous involvement of the viscera than the controls. Westenrijk thought that young bacilli should be used in preparing vaccines, that they should be killed by gentle heat, and that the dosage should be moderate. He ascribed Uhlenhuth's negative findings (*see* 63–65) to excessive doses of vaccine.

Westenrijk also emphasized the importance of choosing a favorable route of administration, and reported the subcutaneous one to be superior in his experience to the intravenous one, although the titer of complement-fixing antibodies was higher after vaccination by the latter. In subsequent studies of rabbits vaccinated by repeated inhalation of aerosols of heat-killed tubercle bacilli, and later challenged with living virulent bacilli by the same technique, Westenrijk (1933, 1936) again found evidence of heightened resistance in the vaccinated animals (144, 145). It is interesting to note that Westenrijk favored a nonliving vaccine against tuberculosis for use in man despite his conviction that living, attenuated vaccines could provide a higher degree of immunity. His choice was dictated by a consideration of the greater safety of killed vaccines and the fact that, although somewhat inferior, the immunity bestowed by nonliving vaccines was still clinically "practical."

Freund (1932) gave repeated subcutaneous injections of heat-killed virulent tubercle bacilli to guinea pigs, and infected them ten days after the last injection by the intradermal route (146). At intervals of one to sixty-seven days after challenge, sample animals were killed, and the number of living tubercle bacilli in their spleens and lymph nodes was determined by cultural methods. Positive cultures were obtained from nonvaccinated control animals sooner than from the vaccinated ones, thus illustrating a retardation of the disease process.

Work with a nonliving vaccine designated "AO" was described by Arima in 1933 (147). The vaccine was prepared by growing tubercle bacilli in saponin-containing medium, and then storing the cultures in the cold until all bacilli were dead. Several hundred children in Germany and Japan who were exposed to tuberculous infection at home were vaccinated with this preparation. Over an observation period of two and one-half years, only one of these children developed clinical tuberculosis. "AO" was also claimed by Arima to have shown therapeutic activity in tests involving several hundred thousand "tuberculous patients" in Japan, but his description of the symptomatology of supposedly tuberculous disease—among the cardinal symptoms are included headache, gingivitis, melancholy, and dismenorrhea—leaves some doubt concerning the actual number of tuberculous patients treated and benefited by the preparation.

Hughes (1933) found that the serum of rabbits which had been injected with heat-killed tubercle bacilli possessed phagocytosis-enhancing properties of the same order as seen in animals infected with living tubercle bacilli, although the "bacteriotrophic substances" appeared earlier in the latter (148).

An investigation of the prophylactic and therapeutic activity of some of Anderson's fractions of tubercle bacilli (149) was undertaken by Smithburn (1933) in the hope of finding an immunogenic, nontoxic moiety (150). One of the fractions, obtained by agitating a culture of tubercle bacilli for five months in an alcohol-ether mixture and then pulverizing the dried residue in a ball-mill, proved to have marked immunogenic activity in rabbits. Animals were given repeated subcutaneous or intravenous injections of the defatted material in saline and were infected with bovine tubercle bacilli three months later by the intravenous route. The median survival time of the vaccinated animals exceeded that of the controls by almost 50 per cent.

Also working with rabbits, Soper, Alpert, and Adams (1933) found three intramuscular injections of 1.5 mg. each of a boiled suspension of an "S" type of bovine culture of tubercle bacilli to bestow a greater degree of protection than a single subcutaneous injection of a large quantity (1.5 mg.) of living BCG (151). Ten to 19 snuffles-free animals were employed in each vaccination

group. Experimental infection took place forty-two to forty-six days after vaccination and was performed by an intrameningeal introduction of virulent tubercle bacilli. The number of animals which failed to become tuberculous was greater in the killed-vaccine–vaccinated group than in the living BCG one, and those animals of the former group which did develop tuberculosis outlived the diseased animals of the latter.

Thomas (1933) vaccinated rabbits with heat- and formol-killed tubercle bacilli (152). Formol was employed as a killing agent in view of Ramon's observations that formaldehyde reduces the toxicity of certain toxins without destroying their antigenicity (153, 154). Thomas found that both vaccines rendered the animals tuberculin skin hypersensitive; but, whereas the preparation sterilized by heating at 70°C. for one hour increased the postinfection life of the animals by almost 50 per cent over that of a control group, the formol-treated preparation elicited no immunity.

Goodwin and Schwentker (1934) vaccinated 73 young children intramuscularly with tubercle bacilli which were killed by boiling for one hour (155). Four weekly injections of 0.03 to 0.1 mg. were given. Abscesses occasionally developed at the site of injection but, although these were quite large in several instances, they were painless and characterized by a minimal degree of inflammation. Fifty of the 73 vaccinated children were observed for a period of time exceeding one year. Two of these children developed tuberculosis, one with a fatal outcome. In contrast, a group of 196 children similarly exposed to tuberculous infection from familial sources, and not vaccinated, had a tuberculosis mortality rate of 4.6 per cent over the same period of time.

Aronson (1935) vaccinated guinea pigs subcutaneously with 5 mg. of living BCG or 30 mg. of heat-killed bovine tubercle bacilli (156). The animals were infected intratracheally six weeks later with 100 virulent bacilli of bovine origin, and were sacrificed and examined six weeks after challenge. The animals which had received living BCG showed less systemic tuberculous involvement and fewer tubercle bacilli in organ sections than the unvaccinated controls. The killed vaccine provided no such state of heightened resistance. However, the vaccinating dose was very large in this experiment—30 mg.—and may well have exceeded the optimal quantity of non-living vaccine.

Branch and Enders (1935) vaccinated guinea pigs by three injections of 3 mg. (wet weight) each of heat- or formol-killed tubercle bacilli (157). Different groups of animals received the vaccines by different routes, each group consisting of fifteen animals. One month later, all animals were infected subcutaneously with 1,000 virulent bacilli. The vaccinated animals survived longer than the unvaccinated controls, the intramuscular route of vaccination proving to be the most effective. Several vaccinated animals remained entirely free of tuberculous disease. The independence of acquired immunity from tuberculin skin hypersensitivity was indicated by the fact that many of the protected animals remained tuberculin negative after vaccination.

Coulaud (1935) found that heat-killed tubercle bacilli administered intramuscularly or subcutaneously in a paraffin suspension doubled the longevity of rabbits and guinea pigs after tuberculous infection by the subcutaneous route five to twenty weeks after vaccination (158). Daranyi (1935) found that the degree of protection elicited in guinea pigs by subcutaneous vaccination with 2 to 4 mg. of heat-killed tubercle bacilli was inferior to that elicited by living BCG (159). However, Daranyi evaluated the degree of protection bestowed by the vaccines solely on the basis of the extent of tuberculous disease seen in animals sacrificed at intervals after infection (by inhalation of tubercle bacilli). This method of evaluation of a vaccine has been found to be frequently misleading (*see* 167) because the size and appearance of tuberculous lesions at a given time are not always indicative of the animal's ability eventually to retard or ameliorate a fatal outcome of the infection.

Lagrange and Lagrange (1935) reported that the survival time of massively infected guinea pigs was more than doubled by treatment before *and* after infection with tubercle bacilli killed by heat and formol (160). When the killed bacilli were given only after infection, there was no protection, thus indicating that the efficacy of the pre- and postinfection treatment was at least partially due to a prophylactic effect. Pregnant females could not be protected by this treatment.

Toda and his co-workers (1935) claimed to have demonstrated immunogenic activity by a tuberculo-phosphatide fraction, and that the protection induced was not accompanied by the development of tuberculin skin hypersensitivity (161).

### 1936–1940

Berg (1936) reported a degree of protection in guinea pigs against experimental tuberculosis as a result of vaccination with tubercle bacilli killed by heat or dying spontaneously after prolonged incubation (162), but the high incidence of intercurrent disease and the small number of animals which were employed reduce the significance of his observations. Tubercle bacilli killed by boiling for thirty minutes were found by Corper, Damerow, and Cohn (1936) to be ineffective in retarding the progress of subsequent tuberculous infections in guinea pigs and other animals (163). Full experimental details were not provided in this communication, but it appears that the amounts of nonliving vaccine given were very small. Moreover, the method of killing was not likely to preserve fully the immunogenic potentials of the bacilli.

Convincing experiments with a nonliving vaccine, involving more than 130 guinea pigs, were reported in 1936 by Ferguson and Cannon (164). The vaccine, consisting of phenol-killed human tubercle bacilli, was given in one or two intratracheal administrations of 0.1 mg. each. The animals were infected one to three months later by the same route, the workers hoping to demonstrate the effect of direct "immunization of the portals of entry" of the invading bacilli. The average survival time of the vaccinated animals after infection was found to be double that of the unvaccinated controls (112 versus 59 days). In other experiments, animals were sacrificed and examined at intervals after infection; the vaccinated guinea pigs had considerably less extensive and less disseminated tuberculosis.

A study directed specifically toward the removal of the toxigenic components of killed tubercle bacilli was undertaken by Machebocuf and Dieryck (1936) (165). Living tubercle bacilli were extracted in the cold with water, acetone, and ether. The residue, dissolved in water, was nontoxic for rabbits and prolonged their survival after challenge infection. The results of extensive vaccination experiments with killed tubercle bacilli, employing 15 to 20 guinea pigs in each vaccination group, were communicated in 1937 by Boquet and Laporte (166). Heat-killed vaccines were found to be more effective in retarding the course of challenge infections than formol-killed ones, and vaccines prepared from "S" cultures of bovine tubercle bacilli were superior to those made from "R" cultures. Repeated injections of vaccine gave better protection than a single vaccination, but emulsification in a lanolin-oil adjuvant did not enhance its efficacy, probably because the quantity of vaccine employed, 10 mg., was large and likely to induce systemic toxicity when given in an oil adjuvant (*see* 237). Boquet and Laporte's study thus again underlines the importance of the techniques of preparation and administration in obtaining positive results against tuberculosis with nonliving vaccines.

Among the most persuasive experiments which have shown the immunogenicity of nonliving vaccines are those reported in 1937 and 1938 by Opie and Freund (167, 168). The nonliving preparation studied most extensively by them consisted of bovine or human tubercle bacilli heated for thirty minutes at 60° or at 100°C. Rabbits of different breeds were employed as experimental animals. Postinfection survival time was taken as the measure of protection in most experiments; estimation of the extent of tuberculous lesions of animals sacrificed at intervals after infection was considered an unsatisfactory criterion of immunity: "This procedure may give doubtful results because numerous observations . . . . have shown that lesions may be more extensive, for a time at least, in previously sensitized than in control animals, whereas at a later period the lesions diminish with healing" (167). Rabbits vaccinated by repeated subcutaneous or intradermal injection of small quantities of heat-killed bacilli showed a high degree of protection against an intravenous challenge with bovine tubercle bacilli sufficiently great to kill all control animals. In some experiments, the vaccinated animals not only outlived the controls, but frequently remained completely free of tuberculous disease.

The protective activity of the heat-killed vaccine was enhanced considerably by administering it together with heated horse serum, the degree of the protection thus obtained being of the same order as that given by living BCG. (It is interesting to note that Freund and Opie found that the addition of *unheated* serum *reduced* the immunogenicity of the killed bacilli, thus corroborating identical findings by Streng and Ryti (142).) Neither heat-killed cultures nor culture filtrates of several other microorganisms or alum had an adjuvant effect. When large challenge doses were given, of the order of 1,000 times the minimal lethal dose, the degree of protection

bestowed by the vaccines was much reduced, possibly, in the opinion of the authors, because of systemic hypersensitivity reactions. Such massive infection is almost exclusively a laboratory phenomenon, however, and cannot be looked upon as a practical exception to the usefulness of nonliving vaccines.

Opie and Freund also referred to limited human trials which had been initiated by them, and concluded that their animal as well as human experiments "indicate that heat-killed tubercle bacilli may be substituted for the living attenuated microorganisms in the attempt to increase resistance against tuberculous infection" (167). Both living and killed vaccines stimulated the production of circulating antibodies, but the investigators found that "there is no correlation between the intensity of sensitization or the titer of antibodies, on the one hand, and resistance to infection on the other" (168).

The results of the human trial initiated by Opie and Freund with heat-killed vaccine (167) were reported in detail by Opie, Flahiff, and Smith (1939) and by Wells, Flahiff, and Smith (1944) (169, 170). The trial was conducted among 2,500 patients, most of them nonwhite, in a mental institution in British Jamaica, where living conditions were poor and tuberculosis morbidity and mortality rates very high. Most of the vaccinated patients received one or two simultaneous intradermal injections of 0.2 to 0.3 mg. of heat-killed tubercle bacilli of bovine origin (Ravenel strain). With this dose, no untoward local reactions developed. The tuberculous disease and death rates fell sharply among the vaccinated patients during the first eighteen months (169); over the full observation period, extending to more than ten years, the tuberculosis mortality of nonvaccinated persons who were tuberculin negative on admission to the hospital was 17.9 per cent, and that of initially tuberculin-negative inmates who received the vaccine, 10.5 per cent. This difference is statistically significant, and provides a clear demonstration of the efficacy and safety of a killed vaccine in man.

Pagel (1937) reported the protection of guinea pigs against intradermal infection with virulent tubercle bacilli by repeated vaccinations prior to infection with heat-killed tubercle bacilli (171). Injections of peptone broth alone also elicited some protection. Not too surprising, in view of the fact that they received eight to ten intraperitoneal injections over a period of several weeks, many of Pagel's animals succumbed to the vaccination.

Saenz (1937) vaccinated guinea pigs subcutaneously or intramuscularly with 10-mg. quantities of heat-killed tubercle bacilli suspended in vaseline, and subsequently infected them with virulent tubercle bacilli by the intramuscular, subcutaneous, or intradermal routes (172, 173). Vaccination resulted in an appreciable retardation of the spread of the disease. Saenz thought tuberculin sensitivity and immunity to be independent phenomena because vaseline enhanced the sensitizing powers of killed tubercle bacilli to a greater extent than their immunizing powers (173).

The use of the mouse as an experimental animal in extensive tuberculosis vaccination studies was reported by Schwabacher and Wilson in 1937 (174). Human tubercle bacilli were killed by heating for ninety minutes at 66°C., or by autoclaving. More than 600 animals were vaccinated subcutaneously or intravenously by two to twenty successive injections of killed vaccine or of living BCG. Challenge infection was administered by the intravenous route two to four weeks after the last vaccination. The animals were permitted to live to term and were then examined at autopsy. The heat-killed and living vaccines were found equally effective in prolonging the life of the infected animals, but this heightened resistance was demonstrable only when a large challenge was employed (10- to 100-million virulent tubercle bacilli). With smaller challenge doses, a chronic rather than an acute tuberculous disease ensued, and little evidence of vaccination-induced immunity could be found. The writers concluded from these observations that vaccination acts only to retard the growth of tubercle bacilli in tissues and that, when infection of a natively highly resistant animal, such as the mouse, is small, the naturally slow process of the disease overshadows any artificially induced immunity. (It should be noted that the opposite effect has been reported by many investigators when employing the guinea pig as an experimental animal: a killed vaccine could protect against a moderate, but not a very large, challenge infection. Differences in the pathogenesis of tuberculosis in different animals must thus be taken into consideration in the design and interpretation of vaccination experiments.)

To rule out the possibility that the effects of

desensitization to tuberculoprotein on the course of tuberculous disease are not due to an *active* immunization, Follis (1938) again attempted to elicit immunity in guinea pigs by vaccination with Old Tuberculin (175). An examination of the organs of animals vaccinated subcutaneously with tuberculin, then challenged with living tubercle bacilli, and sacrificed forty-four days afterward provided no evidence of a heightened resistance.

Hensel (1938) vaccinated 21 guinea pigs with vaseline or lanolin suspensions of tubercle bacilli killed by heating for two hours at 65°C., and infected them six months later by the intradermal route (176). All but one of the vaccinated animals were found to be free of tuberculosis upon post-mortem examination three months after challenge, while 9 of 10 control animals showed tuberculous involvement of lymph nodes and spleen. Here, again, it must be considered as quite possible that this unusually high degree of protection resulted from a mechanical expulsion of the infecting bacilli as a result of a local Koch phenomenon (*see* 188). Van Deinse (1938) injected rabbits intramuscularly with a paraffin mixture containing 1 mg. of powdered, heat-killed avian tubercle bacilli (177). This vaccination influenced favorably the survival time of the animals following an intravenous injection of living avian bacilli five weeks later. However, when the vaccination-challenge interval was six months, the animals succumbed more rapidly to a "toxi-infection type Yersin," because, in Van Deinse's opinion, of the development of pronounced hypersensitivity.

Griffith and Glover (1939) killed bovine tubercle bacilli by several methods: exposure to formalin, high concentrations of glycerin, and heat (178). Groups of 16 guinea pigs each were vaccinated with the different preparations, each animal receiving nine injections at intervals, the first 4 by the intraperitoneal route and the last 5 subcutaneously. They were infected fifty-one days after the last vaccination by intradermal injection or conjunctival instillation of virulent bovine tubercle bacilli. The killed vaccines were found to have "diminished the extent and severity of the generalized tuberculosis in only 50 per cent of the guinea pigs," compared to a control group. A group of animals which had received living BCG did not show a much more pronounced degree of immunity, however. The glycerin-killed preparation was found to be the least toxic.

A retardation of the visceral spread of tuberculous disease was observed by Haefliger (1939) in guinea pigs vaccinated subcutaneously with 5 mg. of steamed bovine tubercle bacilli in vaseline, and infected six weeks later by the intradermal route (179). Haefliger considered the protection by living BCG to be superior, however. Smithburn and Lavin (1939) were unable to protect guinea pigs by intracerebral vaccination with 0.01 mg. of ultraviolet-killed tubercle bacilli against virulent infection by the same route (180). A group of 4 guinea pigs vaccinated with larger doses of ultraviolet-killed bacilli by the subcutaneous route, and challenged intracerebrally, also showed no increase in survival time relative to 5 control animals.

Toda and Murata (1939) vaccinated groups of 5 to 10 guinea pigs each with intact heat-killed tubercle bacilli or various bacillary fractions (181). A polysaccharide fraction reduced the severity of tuberculosis in animals infected intradermally seven days after vaccination without causing them to become tuberculin skin sensitive. A combination of this polysaccharide fraction with intact killed tubercle bacilli elicited a degree of immunity similar to that given by living BCG. (A similar enhancement of the immunizing activity of certain fractions of tubercle bacilli by adding small quantities of intact killed tubercle bacilli has also been observed by Weiss and others (*see* 233, 238). Desensitization of animals made tuberculin positive by some of the preparations did not reduce their acquired resistance, and the workers therefore inclined to the view that "there can be a state of immunity without consequent skin allergy."[11]

Damerow (1940) found no evidence of protection in small numbers of guinea pigs vaccinated subcutaneously with 1-mg. quantities of tubercle bacilli boiled for thirty minutes and challenged by the same route one month later. His criterion of protection was the degree of tuberculous involvement of the organs of animals sacrificed two months after infection (182). Another negative evaluation of killed antituberculosis vaccines was published in the same year (1940) by Liebow, Burn, and Soper (183). These investigators vaccinated large numbers of guinea pigs with

[11] "Das es eine Immunität ohne Gefolge von Hautallergie geben kann."

living BCG or with heat-killed "S" and "R" variants of human tubercle bacilli, and challenged them two months later by an intramuscular injection of virulent human bacilli. The killed vaccine gave no survival prolongation, whereas living BCG did. The experiment is of little importance, however, because 25 to 60 per cent of the animals died from other causes, largely infectious in nature, *prior* to the tuberculous challenge infection, and the pre-challenge mortality among the animals given the killed vaccine was considerably higher than among those vaccinated with living BCG.

### 1941–1945

A statistically analyzed and significant study of immunization of rabbits and guinea pigs with nonliving avian tubercle bacilli and BCG and with bacillary extracts was reported by Widström in 1941 (184). The criterion of immunity in most of the experiments was survival time after infection. Widström found that moderate doses of bacilli ground in a ball-mill until their structural integrity was destroyed gave a high degree of survival prolongation; larger doses of the same preparation were ineffective. Aqueous extracts of the ground bacilli were more effective than the primary material; a lipoidal extract was ineffective but the residue retained immunogenic activity. Treatment of bacillary powders with acid or alkali (pH 1.9 or 10.2) at room temperature enhanced their vaccinating powers. The independence of tuberculin skin sensitivity and protective immunity was again indicated in this experiment by the ability of some bacillary fractions to induce independently one or the other of these responses.

Potter (1942, 1943) killed avian and human tubercle bacilli by incubating them for several weeks anaerobically and studied the immunizing properties of the "asphyxiated" bacilli in rabbits (185, 186). The nonviability of the vaccine was shown by the failure of large quantities to induce progressive disease in pigeons. Five subcutaneous injections of 13 mg. (wet weight) each of the dead avian bacilli increased the survival time of animals infected intravenously with live avian tubercle bacilli four and one-half or twelve months after vaccination (185). In another experiment, rabbits were similarly vaccinated with "asphyxiated" human tubercle bacilli and were challenged intravenously with live human bacilli four months later. Whereas 25 of 31 nonvaccinated control rabbits developed gross tuberculous disease, only 4 of 33 vaccinated siblings presented any evidence of tuberculosis on sacrifice and postmortem examination eight to ten months after infection (186). Potter emphasized the importance of dosage in the use of nonliving vaccines (in comparing them to living ones, he wrote: "For purposes of comparative study the largest well-tolerated doses rather than equal doses might well be employed."), and supported Opie and Freund's (167) choice of rabbits as experimental animals in tuberculosis vaccination studies because their "diseases resemble human tuberculosis in virulence far more than the highly fatal guinea pig infections."

### 1946–1950

Raffel (1946) found repeated injections of large quantities of heat-killed tubercle bacilli or several bacillary fractions (totaling 50 to 100 mg.) ineffective in protecting guinea pigs against massive experimental infection (187), and concluded in a later discussion (1953) that nonliving vaccines are incapable of protecting against tuberculosis (*see* 10).

Choucroun (1947, 1949) undertook a chemical fractionation of tubercle bacilli, and studied the immunogenic and allergenic activities of several fractions (189, 190). Small quantities of a nonsensitizing lipocarbohydrate moiety ("PMKO") gave "a definite degree of acquired resistance in guinea pigs," as judged by prolongation of survival after challenge infection (190). The prophylactic and therapeutic activities of a "carbohydrate-lipoid" fraction of the H37Rv strain of tubercle bacilli were also reported in 1947 by Kropp and Floyd (191). These investigators vaccinated groups of 12 to 20 guinea pigs by repeated subcutaneous injections of the fraction over an eight-week period. Tuberculin skin hypersensitivity was not evoked. Five days after the last injection, the animals were infected intramuscularly with virulent human tubercle bacilli. The vaccinated animals survived considerably longer than nonvaccinated controls. Kropp and Floyd also claimed a beneficial effect for their material in the *treatment* of guinea pig and human tuberculosis.

In presenting the attitude of the U.S. Public Health Service in 1947 on BCG vaccination in the United States, Hilleboe expressed the opinion

that "further research is strongly recommended to determine the efficacy of the vaccination and also to attempt to develop a vaccine composed of dead bacilli" (192), thus indicating that, in some quarters at least, the possibility of a nonliving vaccine has not been discounted during recent years.

Olson, Habel, and Piggott (1947) employed ultraviolet light to kill BCG and human tubercle bacilli (193). Large groups of guinea pigs, from 48 to 60 animals per group, were vaccinated by three intraperitoneal injections of 5 ml. each of the irradiated suspensions at one-week intervals. Other animals received one or three intraperitoneal injections of living BCG. All were infected intraperitoneally with human tubercle bacilli one month later and were permitted to live to term. The ultraviolet-killed vaccine made from human tubercle bacilli was as effective in prolonging survival time as was a single vaccination with living BCG (animals receiving three injections of living BCG showed little immunity). In contrast, the ultraviolet-killed preparation consisting of BCG was ineffective, and the writers reported that heat-killed vaccines consisting of either human bacilli or BCG also failed to bestow a degree of heightened resistance. The type of bacilli and the method of killing employed in preparing a nonliving vaccine were thus again shown to be, in the hands of some workers, of paramount importance.

Ultrasonic vibrations were used by Kress (1948) to kill a suspension of virulent human tubercle bacilli (194). A few guinea pigs vaccinated subcutaneously with the killed suspension, and challenged six weeks later by the intranasal introduction of virulent tubercle bacilli, failed to develop tuberculosis. The experiment lacked adequate controls, however, and cannot be considered to have much significance.

Oka, Yamada, and Niitsu (1949), Oka, Yamada, Konno, Shoji, and Sugawara (1950), and Yamada (1952) vaccinated guinea pigs by scarification or subcutaneous injection of heat-killed tubercle bacilli suspended in paraffin and infected them subcutaneously several weeks later (195–197). The killed preparation gave the same degree of immunity as living BCG, as judged by the extent of tuberculous involvement of the viscera of animals sacrificed at intervals after injection. Vaccination with saprophytic mycobacteria gave no protection. Children vaccinated with the killed preparation by scarification did not develop extensive local lesions, even when the vaccine was administered a second time (197).

Ultraviolet-killed vaccines were subjected to intensive investigation by three groups of investigators in 1949 and 1950 and were independently found to possess a relatively high degree of immunogenic activity. Paterson, Crombie, and Coles (1949) vaccinated groups of 50 guinea pigs each with ultraviolet-killed vole bacilli and with living BCG (198). The vaccines were administered subcutaneously in two injections four weeks apart, and challenge infection took place by the same route four weeks after the second vaccination. The average postinfection survival time of a nonvaccinated control group was thirty-three days; of the group given living BCG, seventy-three days; and of the group given the irradiated vaccine, sixty-four days. Whereas 50 per cent of the animals which had received two injections of 10 mg. (moist weight) each of living BCG developed local abscesses, often with sinus formation, only 4 per cent of those given the killed vaccine (a total of 5 mg., moist weight) developed local lesions, and then only of a "trivial" nature.

Milzer, Levinson, and Lewis (1950) found ultraviolet-killed H37Rv to be slightly superior to living BCG in dba-strain mice (199). The animals were given four subcutaneous injections of 0.5 mg. (wet weight) each of the killed suspension at weekly intervals and were challenged thirty days later by subcutaneous and intraperitoneal injection of living H37Rv bacilli suspended in gastric mucin. The animals were sacrificed one month later, and the extent of their tuberculous disease was compared with that of the nonvaccinated controls and of animals vaccinated with living BCG. The disease process in the vaccinated animals was significantly retarded. Storage of the irradiated vaccine in a 1:10,000 dilution of merthiolate for one year did not reduce its efficacy.

In a series of experiments, Sarber, Nungester, and Stimpfert (1950) vaccinated groups of 55 to 86 guinea pigs by three intraperitoneal injections of 5 mg. each of strains of virulent human tubercle bacilli or BCG killed by ultraviolet irradiation, or with living BCG (200). The animals were infected intraperitoneally three to four weeks later; those surviving more than two to four months after challenge were sacrificed, and the extent of disease was estimated by the Feldman Index method. The killed human

bacilli effected a degree of protection of the same order as living BCG, but the irradiated BCG vaccine gave little protection—a result identical with that obtained by Olson and his co-workers (193).

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# SOME EFFECTS OF MECHANICAL RESPIRATORS UPON RESPIRATORY GAS EXCHANGE AND VENTILATION IN CHRONIC PULMONARY EMPHYSEMA[1, 2]

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## INTRODUCTION

It is well known that in chronic obstructive pulmonary emphysema there are three fundamental disturbances in pulmonary function: (*1*) gross impedence to air flow in and out of the lungs, (*2*) uneven distribution to the alveoli of the blood returning to the lungs, and (*3*) uneven distribution of the air to the alveoli. In the less severe phases of the disease, ventilatory dysfunction exists without disturbed gas exchange. In the more severe forms of the disease, ventilatory insufficiency is coupled with gross disturbances of distribution of air and blood to the alveoli, resulting in anoxia and hypercapnia. The latter may be present constantly in such patients or may occur only intermittently when acute pulmonary infections are superimposed upon the already diseased lung. When anoxia and hypercapnia are constantly present and of moderate to severe degree, circulatory complications may appear in the form of pulmonary arterial hypertension, increased blood volume, polycythemia, and right heart failure (1–5).

The management of the emphysematous patient during acute exacerbations of cardiopulmonary insufficiency has been dealt with in recent papers, and the measures used to combat the underlying disease process and the acute cardiopulmonary dysfunction have been described (6–8, 22). The usefulness of one or another mechanical respirator in aiding ventilation has been pointed out by numerous writers (9–23).

Although it is reasonable to assume that the use of respirators might be beneficial in obstructive emphysema by promoting better alveolar ventilation and thus better respiratory gas exchange, factual physiologic data which demonstrate the effect on ventilation and gas exchange of one or another such respirator are meager (8). Some reports for example, offer only lung volumes and clinical evaluation as evidence for or against the effectiveness of this therapy (24); others have measured lung volumes and the arterial blood oxygen saturation but not the carbon dioxide content or tension (17, 19, 21). Finally, the inspired gas mixture usually contained either high concentrations of oxygen and/or some form of bronchodilator (17, 19, 20, 23), thus making an assessment of the effects of the respirator alone impossible. Data which demonstrate the duration of any effects produced by such respirators are also few. This paper presents a study of the effects of four different respirators on the ventilation and gas exchange of patients with chronic obstructive pulmonary emphysema as observed for various periods of time. All studies were carried out with the subjects breathing from gas cylinders containing gas of ambient air composition, that is to say, no studies were made using pure oxygen or high concentrations of this gas. The patients also did not receive any bronchodilators during the studies; hence, the results could be assigned to the effect of the respirator itself.

## MATERIAL AND METHODS

A total of 43 studies were carried out on 17 patients using four different respirators. Four patients were studied with each of three different respirators, 6 patients with two and 6 with one. One study was carried out on one normal person.

The 17 patients studied were, in every instance, considered to have chronic obstructive pulmonary emphysema, not only by clinical evaluation but also on the basis of pulmonary function studies. The majority of the group had had pulmonary hypertension at some time in their course and also had been in right heart failure and were classed as having cor pulmonale. All 17 patients showed

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some degree of arterial oxygen unsaturation at rest, some degree of hypercapnia, or both.

Thirty-four so-called *acute* studies were made in which the respirators were used for periods of twenty to one hundred and twenty minutes and the effects on the blood gases were determined. The studies were carried out with the patients fasting and rested. Resting levels of pulse and respiratory rates were measured, and control arterial blood samples at rest were obtained. In the early studies, ventilation was not measured until later; when it became apparent that the changes noted in the blood gases probably bore more than a casual relationship to the ventilatory changes obtained, minute ventilations were also measured. A second arterial blood sample was obtained at the end of each study and before the actual discontinuation of the use of the respirator.

Minute ventilations were obtained by collecting the expired gases in a Tissot spirometer. The arterial blood samples were analyzed for pH, carbon dioxide content, oxygen capacity, and oxygen content by standard techniques, and values of carbon dioxide tension ($PaCO_2$) and oxygen saturation were calculated from the analytic results.

In the 7 *chronic* studies the respirator was kept at the patient's bedside and was used for two to fifteen hours a day for several successive days. Arterial blood samples were obtained at rest at the start of each trial, before the patient had used the respirator. At the conclusion of the study period, which varied from four to twenty-one days in duration, blood samples were again taken. These second blood samples, however, were never drawn sooner than twelve hours after the last use of the respirator. No ventilation studies were performed in this group.

The respirators used were the Bennett pressure therapy unit,[4] a pneumatic balanced resuscitator (referred to as the PBR and also called Pneophor),[5] a Huxley cuirass respirator,[6] and a standard Drinker tank respirator. The operation of each of these is briefly outlined below.

*The Bennett apparatus* is a flow-sensitive pressure-triggered device, which is capable of delivering air through a reducing valve from a cylinder of compressed air to the face mask at a maximal instantaneous flow rate of 130 liters per minute, and which can exert a peak face-mask pressure of 24 cm. of water. When the unit is operating, the flow-sensitive valve is opened by the initial inspiratory effort of the patient and remains open as long as air flows into the face mask. When the point of maximal inspiration for the pressure-level set is reached, air flow ceases, the valve closes, and expiration takes place through a separate, low-resistance, expiratory valve. *With this respirator the patient must initiate the inspiratory phase of each respiratory cycle; otherwise no gas flow occurs.* The rate at which a complete respiratory cycle is accomplished, or the cycling time, depends on the rate of air flow and the total lung capacity of the user. The rate of air flow varies directly with the maximal pressure for which the respirator is set and also with patency of the air ways.

*The pneumatic balanced resuscitator*, or Pneophor, is a pressure-sensitive apparatus which is able to deliver air to the face mask at instantaneous rates up to 40 liters per minute and to achieve peak face-mask pressure up to 27 cm. of water. When in operation, the machine delivers air or any other desired gas mixture from the reducing valve of the gas cylinder through the pressure-sensitive valve until the pressure on the face-mask side of the valve becomes equal to, and thereby balances, the pressure on the other or cylinder supply side; at this point the valve is closed off from the positive-pressure supply in the gas cylinder, allowing expiration to take place through this same valve to the atmosphere or to a gas collecting system. Here, too, cycling time depends on rate of flow of gas and the lung capacity of the user. There is an important difference between the pneumatic balanced resuscitator and the Bennett units, namely, the PBR apparatus does not require initiation of inspiration by the subject in order to start the respiratory cycling. Obviously, in an apneic patient this is of utmost importance.

*The Huxley-Technicon cuirass respirator* consists of a rigid shell of molded plastic, fitted snugly over the anterior thorax and abdomen, so that the space between the inner side of the cuirass and the skin is rendered air tight. The air space is connected to a bellows pump by a large-diameter flexible tube. The bellows pump is driven by a variable speed electric motor and can develop pressures within the cuirass air space from −30 to +10 cm. of water at rates up to 26 respiratory cycles per minute.

*The Drinker tank respirator*, used in one acute study, was in all respects a standard model. This unit consists of a horizontal cylindrical tank in which the patient lies with his head protruding from one end through an annular ring of foam rubber which makes an air tight seal at his neck. At the other end of the tank is a loose leather disc at the center of which a smaller metal disc is attached. This combination acts as a kind of piston which is moved by a motor in and out of the tank, creating cyclic changes in the air pressure within the tank. With an increase in the tank pressure the patient is forced to exhale, and with a decrease in the tank pressure the patient inhales. The pressure can be varied between +10 and −20 cm.

[4] Made by Bennett Respiratory Products, 2230 South Barrington Avenue, Los Angeles 64, California.

[5] Made by Mine Safety Appliance Company, 201 N. Braddock Avenue, Pittsburgh 8, Pennsylvania.

[6] Made by Technicon Company, 215 East 149th Street, New York, New York.

of water, and the cycling speed can be varied from a low rate to about 30 cycles per minute.

In those acute and chronic trials using the PBR in which ventilations were not measured, the peak face-mask pressure was adjusted to +13 cm. of water. In all other trials, no matter what the type of respirator, the maximal pressure was adjusted to +20 cm. of water. The only exceptions to this were during the second, shorter part of the single Drinker respirator study and in the Huxley study with the single normal subject, when peak pressures varied. In the case of the face-mask respirators (Bennett and PBR), the peak pressure would be called "positive" pressure with regard to the effect on the respiratory tree and with the cuirass or tank type of respirator would be "negative" cuirass space pressure but with a "positive"-like pressure effect within the respiratory passages. No other means save that of fixing the level of maximal or peak inspiratory pressure was used to standardize the operation of the respirators.

## Results

### *Acute Studies*

The detailed results of the acute studies, using the Bennett, the pneumatic balanced resuscitator, Huxley, and Drinker apparatuses are given in tables 1 through 3, respectively. The changes in oxygen saturation, carbon dioxide content, carbon dioxide tension, ventilation, and tidal air are given in absolute terms and also as percentages of the initial values.

In addition, the relative or percentage changes have been averaged for each group of patients, using the individual respirators (table 3).

*Acute Bennett studies:* Twelve studies were carried out in 10 patients after periods of twenty to one hundred twenty minutes on the apparatus. In 6 of these 12 trials, measurements were

TABLE 1

THE ACUTE EFFECTS OF THE BENNETT RESPIRATOR UPON RESPIRATORY GASES AND VENTILATION

| Patient | Time on Respirator | Arterial Blood pH | Arterial Blood $O_2$ Saturation Per Cent | | Arterial Blood $CO_2$ Content (Volume Per Cent) | | Arterial Blood $pCO_2$ (mm Hg) | | Ventilation* (L/Min. BTPS) | | Tidal Air (Cc. BTPS) | | Respiratory Rate | Peak Mask Pressure |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $O_2$ Saturation | Per Cent Change | $CO_2$ Content | Per Cent Change | $pCO_2$ | Per Cent Change | Ventilation | Per Cent Change | Tidal Air | Per Cent Change | | |
| | *minutes* | | | | | | | | | | | | *per minute* | *cm. $H_2O$* |
| AP[1]† | Control | 7.39 | 82 | | 62.6 | | 54 | | — | | — | | — | — |
| | 50 | 7.47 | 80 | −2.4 | 57.4 | −8.3 | 42 | −22.2 | — | — | — | — | — | +20 |
| WB | Control | 7.39 | 86 | | 61.8 | | 58 | | — | | — | | — | |
| | 50 | 7.55 | 96 | +11.6 | 52.1 | −15.7 | 33 | −43.2 | — | — | — | — | — | +20 |
| AD1 | Control | 7.42 | 94 | | 61.2 | | 48 | | — | | — | | — | |
| | 120 | 7.58 | 95 | +1.1 | 51.9 | −15.2 | 30 | −37.5 | — | — | — | — | — | +20 |
| AY | Control | 7.39 | 81 | | 61.2 | | 52 | | — | | — | | — | |
| | 120 | 7.44 | 79 | −2.5 | 62.6 | +2.3 | 48 | −7.7 | — | — | — | — | — | +20 |
| AD[2]† | Control | 7.38 | 92 | | 58.3 | | 53 | | — | | — | | — | |
| | 60 | 7.53 | 85 | −7.6 | 52.3 | −10.3 | 28 | −47.2 | — | — | — | — | — | +20 |
| NP | Control | 7.30 | 80 | | 58.2 | | 60 | | — | | — | | — | |
| | 20 | 7.35 | 87 | +8.8 | 54.9 | −5.7 | 50 | −16.7 | — | — | — | — | — | +20 |
| LER | Control | 7.42 | 85 | | 60.6 | | 48 | | 7.8 | | 210 | | 37 | |
| | 30 | 7.47 | 91 | +7.1 | 58.5 | −3.5 | 42 | −12.5 | 8.7 | +11.5 | 360 | +71.4 | 22 | +20 |
| BB | Control | 7.37 | 91 | | 57.2 | | 49 | | 5.4 | | 220 | | 24 | |
| | 20 | 7.49 | 92 | +1.1 | 49.0 | −14.3 | 35 | −28.6 | 19.5 | +262 | 1100 | +400 | 16 | +20 |
| AW | Control | 7.39 | 90 | | 53.2 | | 45 | | 6.9 | | 250 | | 27 | |
| | 50 | 7.36 | 91 | +1.1 | 57.2 | +7.5 | 51 | +13.3 | 7.4 | +7.3 | 460 | +84 | 16 | +20 |
| HAL | Control | 7.43 | 95 | | 49.3 | | 38 | | 5.6 | | 380 | | 21 | |
| | 30 | 7.54 | 99 | +4.2 | 44.5 | −6.9 | 29 | −23.7 | 9.7 | +73 | 690 | +81.5 | 14 | +20 |
| HOL | Control | 7.44 | 88 | | 45.5 | | 37 | | 8.3 | | 300 | | 28 | |
| | 50 | 7.55 | 93 | +5.7 | 39.4 | −13.4 | 26 | −29.7 | 10.6 | +28 | 560 | +86.7 | 24 | +20 |
| AP[2]† | Control | 7.39 | 87 | | 40.5 | | 35 | | 7.2 | | 300 | | 24 | |
| | 40 | 7.45 | 98 | +12.6 | 39.8 | −1.7 | 30 | −14.3 | 10.4 | +45 | 430 | +43.4 | 22 | +20 |

* Ambient pressure and saturated with water vapor.

† Indicates patients who were studied more than once.

## TABLE 2

(A) The Acute Effects of the Pneumatic Balanced Resuscitator (PBR) upon Respiratory Gases and Ventilation

| Patient | Time on Respirator | Arterial Blood pH | Arterial Blood $O_2$ Saturation Per Cent | | Arterial Blood $CO_2$ Content (Volume Per Cent) | | Arterial Blood $pCO_2$ (mm Hg) | | Ventilation* (L/Min. BTPS) | | Tidal Air (Cc. BTPS) | | Respiratory Rate | Peak Mask Pressure |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $O_2$ Saturation | Per Cent Change | $CO_2$ Content | Per Cent Change | $pCO_2$ | Per Cent Change | Ventilation | Per Cent Change | Tidal Air | Per Cent Change | | |
| | *minutes* | | | | | | | | | | | | *per minute* | *cm. $H_2O$* |
| AD† | Control 1 | 7.33 | 77 | | 66.9 | | 64 | | — | | — | | — | — |
| | Control 2 | 7.35 | 71 | | 71.6 | | 69 | | — | — | — | — | — | |
| | | | | +13.0 | | −4.2 | | −25.8 | | — | | — | | |
| | 60 | 7.43 | 84 | | 66.5 | | 50 | | — | | — | | — | +13 |
| NP | Control | 7.36 | 84 | | 64.4 | | 56 | | — | | — | | — | |
| | | | | +6.0 | | −2.2 | | 0 | | — | | — | | |
| | 60 | 7.36 | 89 | | 63.0 | | 56 | | — | | — | | — | +13 |
| BB† | Control | 7.36 | 82 | | 58.2 | | 52 | | — | | — | | — | |
| | 60 | 7.40 | 86 | | 55.6 | | 46 | | — | | — | | — | +13 |
| | | | | +5.8 | | −4.3 | | 0 | | — | | — | | |
| | 120 | 7.35 | 88 | | 55.7 | | 52 | | — | | — | | — | +13 |
| SS | Control | 7.42 | 83 | | 57.3 | | 45 | | — | | — | | — | |
| | | | | +13.3 | | −1.7 | | 0 | | — | | — | | |
| | 90 | 7.42 | 94 | | 56.3 | | 45 | | — | | — | | — | +13 |
| JB | Control | 7.41 | 90 | | 56.4 | | 46 | | — | | — | | — | |
| | | | | +5.6 | | −6.7 | | −13.1 | | — | | — | | |
| | 60 | 7.45 | 94 | | 52.6 | | 40 | | | | — | | — | +13 |
| LER | Control | 7.42 | 82 | | 62.7 | | 52 | | 7.9 | | 210 | | 37 | |
| | | | | +8.5 | | +2.6 | | +15.4 | | +3.8 | | +28.6 | | |
| | 60 | 7.36 | 89 | | 64.3 | | 60 | | 8.2 | | 270 | | 32 | +20 |
| BB† | Control | 7.40 | 92 | | 62.2 | | 52 | | 5.9 | | 230 | | 26 | |
| | | | | 0 | | −9.5 | | −23.1 | | +64 | | +56.5 | | |
| | 60 | 7.48 | 92 | | 56.3 | | 40 | | 9.7 | | 360 | | 27 | +20 |
| AD† | Control | 7.31 | 81 | | 60.6 | | 60 | | 4.7 | | 260 | | 18 | |
| | | | | +14.8 | | −17.2 | | −23.3 | | +121 | | +419 | | |
| | 60 | 7.39 | 93 | | 50.2 | | 46 | | 10.4 | | 1350 | | 30 | +20 |
| AW | Control | 7.33 | 86 | | 55.1 | | 53 | | 8.0 | | 280 | | 20 | |
| | | | | +3.5 | | +3.8 | | +5.7 | | +31 | | +57.2 | | |
| | 50 | 7.32 | 89 | | 57.2 | | 56 | | 10.5 | | 440 | | 20 | +20 |
| DC | Control | 7.33 | 92 | | 52.9 | | 51 | | 5.3 | | 230 | | 23 | |
| | | | | +4.6 | | +1.9 | | −2.0 | | +57 | | +113 | | |
| | 45 | 7.35 | 96 | | 53.9 | | 50 | | 5.8 | | 490 | | 17 | +20 |
| HAL | Control | 7.39 | 95 | | 50.4 | | 43 | | 5.7 | | 390 | | 20 | |
| | | | | −2.1 | | −2.6 | | −11.6 | | +116 | | +162 | | |
| | 30 | 7.43 | 93 | | 49.1 | | 38 | | 12.3 | | 1020 | | 12 | +20 |
| AP | Control | 7.36 | 79 | | 50.0 | | 43 | | 9.5 | | 450 | | 21 | |
| | | | | +8.9 | | −2.6 | | −2.3 | | +6.3 | | +116 | | |
| | 50 | 7.36 | 86 | | 48.6 | | 42 | | 15.5 | | 970 | | 16 | +20 |
| HOL | Control | 7.44 | 84 | | 49.0 | | 38 | | 7.8 | | 300 | | 25 | |
| | | | | +7.2 | | −4.9 | | −2.6 | | +21 | | +23.5 | | |
| | 50 | 7.44 | 90 | | 46.6 | | 37 | | 9.4 | | 370 | | 26 | +20 |
| SCH | Control | 7.46 | 96 | | 46.2 | | 36 | | 6.1 | | 320 | | 19 | |
| | | | | +1.0 | | −8.2 | | −16.6 | | +90 | | +178 | | |
| | 30 | 7.50 | 97 | | 42.4 | | 30 | | 11.6 | | 890 | | 13 | +20 |

(B) The Acute Effects of the Drinker Respirator upon the Respiratory Gases and Ventilation

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| WB | Control | 7.40 | 85 | | 62.4 | | 54 | | 8.5 | | 260 | | 33 | — |
| | 100 | 7.47 | 93 | +9.4 | 59.2 | −5.1 | 46 | −14.8 | 6.2 | −27 | 260 | 0 | 22 | +5 to −16 |
| | 130 | 7.50 | 95 | +11.8 | 57.9 | −7.2 | 42 | −22.2 | 6.1 | −28 | 260 | 0 | 22 | +10 to −21 |

* Ambient pressure and saturated with water vapor.
† Indicates patients studied more than once.

TABLE 3

AVERAGED RESULTS OF ACUTE STUDIES ON THE BENNETT, PNEUMATIC BALANCED RESUSCITATOR (PBR), AND HUXLEY RESPIRATORS

| Type of Respirator | | Number of Cases | Average Length of Run | Average Per Cent Change in Ventilation | Average Per Cent Change in Tidal Air | Average Per Cent Change in $O_2$ Saturation | Average Per Cent Change in $CO_2$ Content | Average Per Cent Change in $pCO_2$ |
|---|---|---|---|---|---|---|---|---|
| | | | *minutes* | | | | | |
| Bennett | With ventilation | 6 | 36 | +71 | +128 | +5.3 | −5.2 | −15.9 |
| | Without ventilation | 6 | 70 | — | — | +1.5 | −8.8 | −29.1 |
| | All cases | 12 | 53 | — | — | +3.4 | −7.0 | −22.5 |
| PBR | With ventilation | 9 | 45 | +57 | +128 | +5.6 | −4.1 | −6.5 |
| | Without ventilation | 5 | 78 | — | — | +8.7 | −3.8 | −7.8 |
| | All cases | 14 | 56 | — | — | +6.7 | −4.0 | −7.1 |
| Huxley | All cases | 7 | 93 | −31 | +2 | −1.9 | −0.8 | −4.1 |

made of ventilation and tidal air as well as of blood gases. In 9 studies (table 1), the arterial oxygen saturation tended to rise after the use of this respirator; the average rise for the group was 3.4 per cent (table 3). However, the rise was significant in only 6 patients (*W. B., N. Per., Ler., Hal., Hol.*, and *A. P.*) In those patients in whom ventilation was measured the average increase in oxygen saturation was 5.3 per cent and average increase in ventilation was 71 per cent. One patient (*B. B.*) showed an increase of 262 per cent. With the exclusion of this patient, the average increase in ventilation would have been 33 per cent. In 10 of the 12 trials the carbon dioxide tension fell significantly. The 2 patients who showed no alteration in carbon dioxide tension (*A. Y.* and *A. W.*) had no change in other blood gas values, suggesting that the respirator was completely ineffective at that time.

*Acute pneumatic balanced resuscitator studies:* Twelve subjects were studied after periods of thirty to one hundred and twenty minutes on the respirator, and 2 were studied twice. Nine trials were made with simultaneous blood gas, ventilation, and tidal air determinations. As may be seen in tables 2*A* and 3, the trend was for a rise in blood oxygen saturation, a significant increase (a change of more than 3 per cent) occurring in ten of the fourteen trials. The average increase in oxygen saturation was 6.7 per cent for the whole group and 5.6 per cent for those patients with ventilation measurements in which there was an average increase of 57 per cent. In contrast, only five of the fourteen trials resulted in a significant fall in carbon dioxide tension and two of these five were just at the borderline of significance (a change greater than 5 mm. of mercury).

*Acute Huxley studies:* Of the 7 emphysematous subjects tested on this device, none derived any amelioration in the abnormal blood gas values after its use (table 3). The study made on a normal subject showed that carbon dioxide tension can be decreased (from 38 to 20 mm. of mercury), and ventilation increased 26 per cent by this ventilator in an individual with normal chest mechanics, although it is not useful as a hyperventilator in the presence of the fixed, immobile chest cage of the emphysematous patient.

*Acute study of Drinker respirator:* The results of the one study made with the Drinker respirator, while the subject was breathing room air, are given in table 2*B*. In the first one hundred minutes of the run, during which period the tank pressure range was +5 to −16 cm. of water, there was a drop in the patient's respiratory rate from his previous resting rate of 32 to 22, the latter being the highest rate attainable with the respirator used. There was no drop in tidal air, but there was a drop in the minute ventilation to roughly 75 per cent of the resting level during unassisted breathing. Nevertheless, there was an increase in the oxygen saturation from 85 to 93 per cent, a rise of 9.4 per cent. There were also decreases in the carbon dioxide content and carbon dioxide tension (−5.1 and −14.8 per cent, respectively). During the last thirty minutes of the study, when the pressure range was increased to +10 to −21 cm. of water, there was a further increase in oxygen saturation to a level of 95 per cent, or an over-all change of 11.8 per cent and further decreases in carbon dioxide content and carbon dioxide tension. In view of the lack of change in the tidal air and the decrease in minute ventilation, the changes in the blood gases cannot be ascribed to an increase in total ventila-

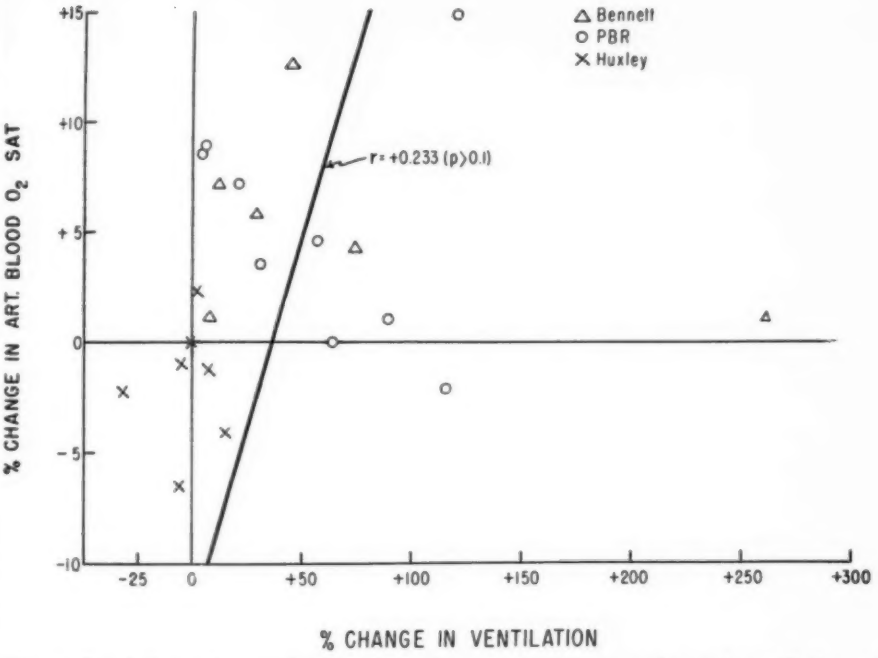


FIG. 1. Correlation and regression line drawn between the percentage changes in ventilation and in carbon dioxide content of the arterial blood in patients with emphysema, using three different respirators, the Bennett (symbolized by the triangles), the pneumatic balanced resuscitator (PBR) (the circles), and the Huxley (the crosses).

tion but must be explained by some other factor, such as better distribution of gas to the alveoli.

*Statistical analysis:* Since it is obvious that changes in minute ventilation or tidal volume must be considered in the interpretation of the blood gas results obtained during use of the respirators, the relationship between changes in ventilation and blood gas changes was analyzed statistically in the 22 patients in whom both values were measured.

In figure 1 the regression line ($r = +0.233$, $P > 0.1$) is plotted for the change in ventilation versus the change in oxygen saturation. The correlation is fair. The regression line intersects the abscissa (coordinate for ventilation change) at a point corresponding to an increase in ventilation of approximately +35 per cent. The slope of the line is positive, so that, when increases in ventilation are greater than the value of the intersept +35 per cent, there is an increase in oxygen saturation; and when ventilation changes are less than +35 per cent, there is a decrease in oxygen saturation.

In figure 2 the regression line is drawn for changes in ventilation and changes in carbon dioxide content. The correlation is obviously good [$r = -0.608$; $(0.01 > P > 0.001)$]. The line intersects the abscissa (ventilation coordinate) at a point corresponding to an increase in ventilation of +25 per cent.

*Summary of acute studies:* In summary, the results of the acute studies show that there is a direct relationship between the change in ventilation produced by a respirator and the resulting change in the levels of the oxygen saturation and the carbon dioxide content. The change in carbon dioxide content showed a closer correlation with the variation in ventilation than did the change in oxygen saturation. When there was an increase in ventilation of more than 25 per cent, there was a decrease in carbon dioxide content. Arterial oxygen saturation did not increase until

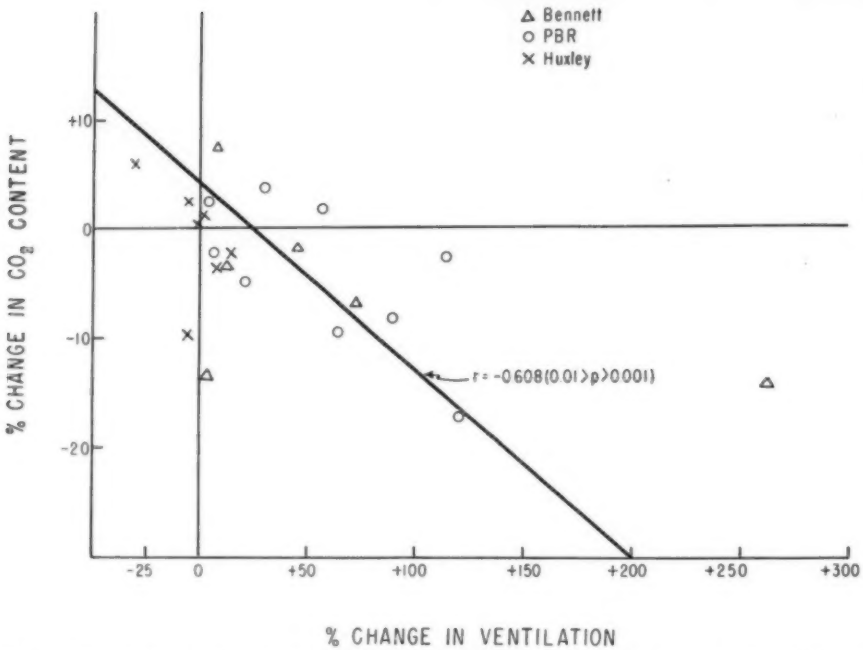


FIG. 2. Correlation and regression line drawn between the percentage changes in ventilation and in arterial blood oxygen saturation in the same patients and with the same respirators as in figure 1.

ventilation rose over 35 per cent. No significant effect on the correlations could be found due to differences in initial level of oxygen saturation or carbon dioxide content. The averaged results of the acute studies (table 3), as well as the correlations, indicated that the PBR and the Bennett are about equal in their effectiveness in increasing the ventilation, and the Huxley is not at all effective. For the same ventilatory change, the PBR is somewhat more effective than the Bennett in increasing the oxygen saturation, whereas the Bennett is more effective than the pneumatic balanced resuscitator in decreasing the carbon dioxide content.

*Chronic Studies*

The detailed results of the seven chronic studies are given in table 4.

The three runs with the Bennett apparatus were of approximately the same duration, six or seven days; but the daily use ranged from two to nine hours. The increases effected in the oxygen saturation were 3.4, 4.6, and 1.2 per cent (average 3.1 per cent); only the first two values were significant changes. In only one subject (*A. D.*) was there a significant decrease in carbon dioxide content and carbon dioxide tension.

The two runs with the pneumatic balanced resuscitator took four and twenty-one days, respectively, with the respirator in use for several periods each day totaling in each case twelve hours a day. The increases in oxygen saturation (+47 and +8.5 per cent) and the decreases in carbon dioxide content and carbon dioxide tension (−8.1, −10.0, −23.4, and −21.4 per cent respectively) were of the same order of magnitude as those from the acute runs, using this apparatus.

The rate at which the changes noted took place is indicated by the serial values obtained in the study on the patient *A. D.*, which show that continual changes in the blood values took place over the whole period of the study.

The two trials with the Huxley were carried out for three and nine days with the respirator in use fifteen and twelve hours a day. In one

TABLE 4

THE CHRONIC EFFECTS OF THE BENNETT, PNEUMATIC BALANCED RESUSCITATOR (PBR), AND HUXLEY RESPIRATORS ON THE RESPIRATORY GASES

| Type of Respirator | Patient | Time | | Arterial Blood pH | Arterial Blood $O_2$ Saturation | | Arterial Blood $CO_2$ Content (Volume Per Cent) | | Arterial Blood $pCO_2$ | | Peak Mask Pressure |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Days | Hours/Day | | $O_2$ Saturation | Per Cent Change | $CO_2$ Content | Per Cent Change | $pCO_2$ | Per Cent Change | |
| | | | | | | | | | | | *mm $H_2O$* |
| Bennett | B. B. | Control | | 7.40 | 87 | | 56.3 | | 48.0 | | — |
| | | 6 | 2-3 | 7.38 | 90 | +3.4 | 55.7 | −1.1 | 50.0 | +4.2 | +20 |
| | A. D. | Control | | 7.40 | 87 | | 59.5 | | 48.5 | | — |
| | | 7 | 9 | 7.42 | 91 | +4.6 | 53.8 | −9.6 | 42.0 | −13.2 | +20 |
| | A. P. | Control | | 7.43 | 83 | | 64.6 | | 50.0 | | — |
| | | 7 | 5 | 7.38 | 84 | +1.2 | 62.5 | −3.3 | 56.0 | +12.0 | +20 |
| Average per cent change | | | | | | +3.1 | | −4.7 | | +0.9 | |
| PBR | A. D. | Control | | 7.35 | 64 | | 67.1 | | 64 | | — |
| | | 7 | 12 | 7.34 | 88 | +47 | 57.5 | −8.1 | 54 | −23.4 | +13 |
| | | 14 | 12 | 7.37 | 85 | | 62.9 | | 56 | | +13 |
| | | 21 | 12 | 7.40 | 94 | | 61.7 | | 49 | | +13 |
| | A. Y. | Control | | 7.40 | 82 | | 63.9 | | 56 | | — |
| | | 4 | 12 | 7.45 | 89 | +8.5 | 57.5 | −10.0 | 44 | −21.4 | +13 |
| Average per cent change | | | | | | +27.8 | | −9.0 | | −22.4 | |
| Huxley | A. D. | Control | | 7.43 | 92.5 | | 62.6 | | 49.5 | | — |
| | | 3 | 15 | 7.42 | 95.0 | +2.7 | 58.3 | −6.8 | 49.0 | −1.0 | −20 |
| | A. Y. | Control | | 7.33 | 74.0 | | 69.0 | | 65.0 | | — |
| | | 3 | 12 | 7.40 | 82.0 | | 66.4 | | 54.0 | | −20 |
| | | 6 | 12 | 7.42 | 84.0 | +8.1 | 64.4 | −11.3 | 52.0 | −20.0 | −20 |
| | | 9 | 12 | 7.39 | 80.0 | | 61.2 | | 52.0 | | −20 |
| Average per cent change | | | | | | +5.4 | | −9.1 | | −11.1 | |

subject (*A. D.*) little change was elicited. In the second patient (*A. Y.*) the carbon dioxide content continued to decrease throughout the study period of nine days. Although the oxygen saturation rose early, it did not continue to improve.

*Long-term chronic studies:* In any evaluation of prolonged or chronic use of respirators, assaying of results would be greatly facilitated if the spontaneous variations in blood gas values due to the disease itself were known over a long period of time. In figure 3 the blood values are plotted of a patient with severe emphysema and periods of marked hypoxia and hypercapnea obtained over several years not only during hospitalization but also on follow-up visits to the clinic. Prior to the respirator studies (using the PBR) the patient had been maintained with regimens which included digitalis, mercuhydrin, bronchodilators, antimicrobials, and phlebotomies. The swings in the blood levels of oxygen saturation, carbon dioxide content, and carbon dioxide tension characteristic of this period of treatment are graphically shown in the plot. When using the respirator, the levels of the blood gases were stabilized at levels much closer to normal; and these levels were maintained for as long as the respirator was used. With termination of its use, rapid return to abnormal levels was noted. A study in a similar patient, using the Bennett apparatus, yielded the same results but is not given in detail here.

Thus, the results of the "long-term" chronic studies show that the PBR and the Bennett respirators can quickly return to nearly normal levels

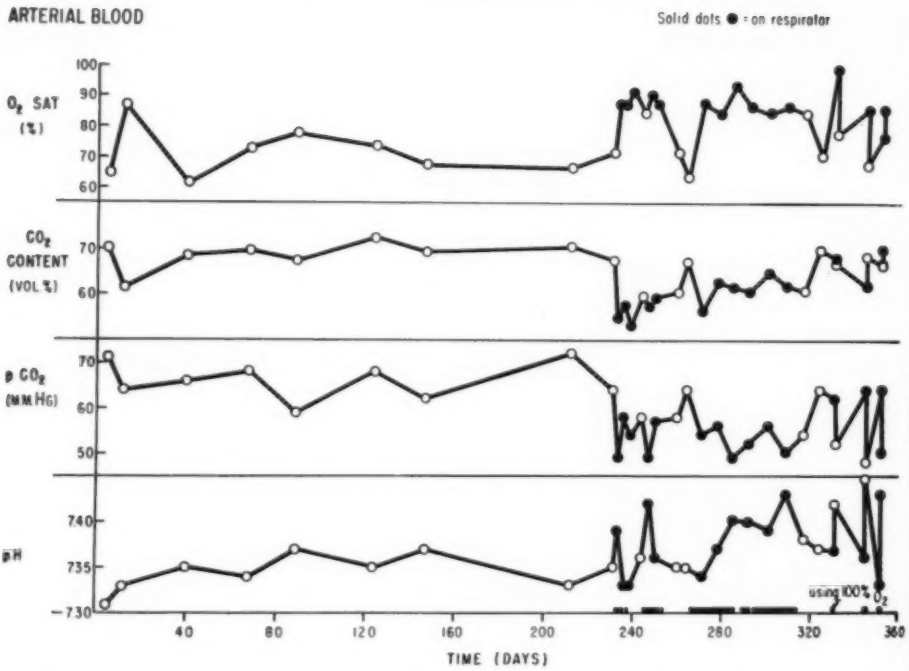


FIG. 3. Values for arterial blood oxygen saturation, carbon dioxide content, carbon dioxide tension and pH, over a period of 360 days in a patient (*A. D.*) with severe emphysema before and during the use of the PBR. The black bars indicate the duration of time the respiratory aid was used, and the solid dots the values of arterial blood gases during its employment.

the abnormal values of arterial blood oxygen saturation, carbon dioxide tension, and carbon dioxide content in the severely emphysematous patients with acute bouts of respiratory failure and that these more normal values can be maintained by the continued use of the respirators.

## DISCUSSION

In normal persons, increases in minute ventilation, by reducing the alveolar carbon dioxide tension and raising the alveolar oxygen tension, reduce the arterial blood carbon dioxide content and raise the oxygen saturation. That a similar effect could be produced in emphysematous patients by the acute or chronic use of respirators has been shown in this study.

Since the results of the acute studies indicate that there is a close correlation between changes in ventilation and arterial blood carbon dioxide, and that there is a less close but none the less significant correlation between ventilatory changes and changes in arterial blood oxygen saturation, one might justifiably ascribe the changes to simple hyperventilation. However, the results of the acute Drinker study, in which a decrease in ventilation was accompanied by an increase in arterial oxygen saturation and a decrease in arterial carbon dioxide, suggest that in some instances better distribution of the inspired mixture may also play an important role (23). A decrease in the work of breathing, as achieved by the respirator, might account for some of this alteration in gas exchange by reducing the oxygen uptake and carbon dioxide production. However, this information was not obtained, so no final conclusion can be reached on these points with the data at hand.

Another interesting fact is that a critical level of ventilation must be achieved by the respirators in order to affect blood gases favorably, since carbon dioxide content is increased and oxy-

gen saturation decreased with ventilatory increases of less than 25 to 35 per cent. Voluntary hyperventilation of such a degree (25 to 35 per cent) would result in sizable changes in blood gases. Therefore, the need to increase ventilation approximately 25 to 35 per cent before producing beneficial blood gas changes indicates that the respirator is less efficient than a similar but voluntary effort and this may be the result of resistance to the machine on the part of the patient. Failure of the patient to allow the respirator to cycle fully, as well as his anticipation of its action (particularly in accelerating the end of the inspiratory part of the cycle) would result in the necessity of overcoming some resistance in initiating expiration, thus making for less efficient ventilation.

These studies also indicate that there was a difference in effectiveness between the three respirators. While the Huxley cuirass is effective in a person with normal chest mechanics, it cannot hyperventilate a severely emphysematous patient with a rigid chest wall. The pneumatic balanced resuscitator and the Bennett respirators produced approximately the same degree of ventilatory change but the PBR influenced oxygen saturation more effectively than the Bennett, and the latter was more effective with regard to the arterial blood carbon dioxide than was the PBR. These differences are probably the result of some factor other than simple ventilation, and may be related to the difference in the mechanical performance of these two apparatuses, a difference which can be shown by the shape of the flow curves obtained in the face masks with each. The Bennett reaches a maximal flow almost instantaneously at the onset of inspiration and maintains it at a plateau until expiration begins, while in the PBR flow rises slowly to a peak at the end of inspiration and then slowly declines during expiration. The two respirators differ in the volume flow; at the same gauge-pressure reading the Bennett has a larger instantaneous rate of volume flow of air than does the PBR. Thus the Bennett, filling the mask and respiratory passages suddenly, flushes out readily ventilatable spaces, hyperventilates these, and lowers carbon dioxide. The PBR, filling the mask and passages slowly, may open up previously closed but perfused spaces and so increase oxygen saturation. In support of this it should be noted that changes in tidal air are larger with the PBR than with the Bennett. Thus, alteration in actual distribution of air throughout the respiratory tree, particularly at the alveolar level, may result with the PBR.

Since this work was done, modifications have been made on the PBR and Bennett respirators, and new respirators of comparable type manufactured by other companies have appeared. The details of construction and operation differ but, unless there is a marked difference in the characteristics of the mask pressure-flow rate, they would presumably produce results similar to those used in this study. The magnitude of the changes would depend in major degree on the extent of the ventilatory changes produced.

The chronic studies indicate that long-term sustained improvement is possible with the PBR and Bennett respirators. Apparently the changes in the blood gases which took place during the periods of use of these two respirators were not fully dissipated while the patients were off the respirators. Thus, the gain of each period of use, added to the gain remaining from earlier periods of use, resulted in steady progress, which was greater for the whole study than for any single period of use. It is worth noting in this connection that the 2 patients, *A. D.* and *A. V.*, studied chronically with these 2 respirators, were the 2 most severely incapacitated patients in any of the studies.

The data derived from the long-term studies are really the most critical demonstration of all in terms of treatment of the severely emphysematous patient, since they indicate that the use of the respirators eliminated the swings in blood gases seen previous to their use, and this stabilization lasted as long as the respirators were used.

In conclusion, these studies demonstrate that certain positive pressure respirators can reduce the elevated arterial blood carbon dioxide content of the emphysematous patient, whether they are used for brief periods or over an extended period of time. This reduction is directly related to the increase in minute ventilation achieved. Similarly, arterial blood oxygen saturation is increased, but the relation to ventilation is less direct, raising the possibility that some other factors such as distribution of gas, for example, may play a role.

Thus, the use of these respirators in the therapy of emphysematous patients with disturbances in gas exchange is rational and is based on demon-

strable beneficial results. Their use for the rapid reduction in levels of carbon dioxide content and increase in oxygen saturation and for maintenance of a lowered carbon dioxide and a raised oxygen saturation is warranted and indicated.


## Summary

The changes in the arterial blood gases in response to the short- and long-term use of four different respirators were studied in 17 emphysematous patients. Ventilation was increased with two respirators and arterial blood carbon dioxide was reduced and oxygen saturation increased with three of the four respirators.

## Sumario

*Algunos Efectos de los Respiradores Mecánicos sobre el Intercambio Respiratorio de Gases y la Ventilación en el Enfisema Pulmonar Crónico*

En 17 enfisematosos se estudiaron las alteraciones observadas en los gases de la sangre arterial a continuación del empleo a breve y a largo plazo de cuatro distintos respiradores. La ventilación aumentó con dos respiradores, y disminuyó el bióxido de carbono de la sangre arterial y aumentó la saturación con oxígeno con tres de los cuatro respiradores.

## Resume

*Certains effets des respirateurs automatiques sur L'échange des gaz respiratoires et la ventilation dans l'emphyseme pulmonaire chronique*

Les variations des gaz dans le sang artériel en réponse à l'emploi à court ou à long-terme de quatre respirateurs différents ont été étudiées chez 17 emphysémateux. La ventilation était augmentée avec deux respirateurs, le $CO_2$ du sang artériel était réduit et la saturation en oxygène était accrue ave trois des quatres respirateurs.


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# CHEMOTHERAPY AND PATHOLOGY OF EXPERIMENTAL PHOTOCHROMOGENIC MYCOBACTERIAL INFECTIONS[1, 2]

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(Received for publication May 8, 1959)

## Introduction

Administration of the currently available antituberculous drugs to patients infected with mycobacteria other than *M. tuberculosis* generally is not accompanied by as favorable a response as that seen in the initial treatment of ordinary pulmonary tuberculosis (1–3). This is undoubtedly related to the fact that the "atypical" mycobacteria are less susceptible than tubercle bacilli to almost all of these agents *in vitro* (4). In addition, the emergence of populations with even greater drug resistance may be induced by serial transfers in drug-containing medium (5) and may also occur in treated patients.

The "atypical" mycobacteria are not uniform in their drug susceptibility, the photochromogens usually demonstrating less resistance than the other strains to isoniazid, amithiozone, and thiocarbanilides (4, 6). Cycloserine uniquely is able to inhibit the growth of almost all mycobacteria equally (4).

Investigation of the effect of drug treatment *in vivo* is hampered by the lack of virulence of the atypical mycobacteria for experimental animals. The photochromogens will usually produce progressive disease in mice, but different strains vary in their degree of pathogenicity and in their response to therapeutic agents (5, 6).

The experiments to be described herein were designed to determine the effect of a number of single and combined drug regimens on a standardized mouse infection produced by one well-known strain of photochromogenic mycobacteria. Since the data regarding survival time and the average amount of gross disease proved to be indecisive, qualitative and quantitative culture techniques finally were utilized to compare regimens.

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[2] This investigation was supported by a grant from the Antituberculosis League of Cleveland and Cuyahoga County, and by a research grant, E 1646, from the National Institutes of Allergy and Infectious Diseases, U. S. Public Health Service.

## Materials and Methods

The strain used for infection was a photochromogen isolated in 1953 from patient *M. B.* (Brownell) at Trudeau Sanatorium. It was described in detail in 1957 (7), and was identified as number 8 (or PS) in the Runyon package of atypical mycobacterial strains for the Cooperative Investigation sponsored jointly by the Laboratory Committee of the American Trudeau Society and the Veterans Administration (8). The culture had been transferred on solid medium at irregular intervals since its isolation.

Infection was accomplished by the injection of 0.1 to 0.15 ml. of culture suspension into a lateral tail vein of the mouse. The organisms were harvested directly from slants of oleic-acid–albumin agar or egg medium which had been seeded with homogenized lungs of control mice from the preceding experiment. The growth was ground lightly in a mortar, suspended in 0.1 per cent bovine albumin in phosphate buffer at pH 6.7, and adjusted by eye to a standard turbidity after allowing the clumps to settle for ten or fifteen minutes. Appropriate dilutions were plated out to determine the number of viable units in the inoculum. The mice were $CF_1$ males, 16 to 29 gm. in weight, purchased from Carworth Farms prior to each experiment.

All drugs except streptomycin were administered by the drug-diet method. The powdered drugs were thoroughly mixed with pulverized mouse pellets in a dry batch mixer and fed *ad libitum*. Streptomycin was given subcutaneously once daily six days a week. Autopsies at the end of each experiment were performed without identification of group or treatment regimen.

### *Preliminary Trial*

A preliminary experiment was undertaken to determine the proper infecting dose and duration of therapy. An inoculum of 4 million viable units was prepared from a young Tween®-albumin liquid medium culture which originated from the stock strain carried on solid medium slants. Therapy was started after a delay of eighteen days and was continued for thirty-five days, after which all survivors were killed. The four treatment regimens, each administered to 10 mice, included cycloserine, 1.0 per cent; hinconstarch, 1.0 per cent[3]; isoniazid, 0.1 per cent; and streptomycin, 5 mg. daily. None

[3] A synthetic polymer of isoniazid, amithiozone, and starch.

of the untreated mice succumbed during the experiment, and there was no significant difference between control and treated groups in the moderate amount of gross disease in lungs and kidneys. It was evident that a more powerful infection and perhaps a longer treatment period were desirable. Having caused the deaths of 3 mice during the first ten days of therapy, 1 per cent of cycloserine in the diet was obviously too toxic.

## Observations

*Experiment I:* The culture used for infection was obtained from the homogenized lungs of an untreated mouse from the preliminary trial. Each mouse received approximately 25 million viable units. Therapy was instituted on day 3 and continued until day 90, when all survivors were killed. Ten drug regimens were evaluated: streptomycin, isoniazid, cycloserine, amithiozone, sulfadiazine, diaminodiphenylsulfone, isoniazid-diaminodiphenylsulfone, isoniazid-amithiozine, isoniazid-streptomycin, and isoniazid-streptomycin-diaminodiphenylsulfone. (*See* table 1 for dosages.) At autopsy, microscopic sections and impression smears for acid-fast bacilli were made from the organs of 2 or 3 mice at random in each group.

Dosages, mortality data, and comparison of gross disease in lungs and kidneys may be seen in table 1. Premature deaths associated with drug toxicity were observed in mice treated with streptomycin and with diaminodiphenylsulfone. Beginning on the second day of treatment, several mice showed an immediate anaphylactic-like reaction after the injection of 5 mg. of streptomycin. Characterized by collapse and respiratory difficulty, the reaction lasted ten or fifteen minutes, and was usually followed by gradual recovery. Two mice died during the acute attack on the fourth day, but after a two-day rest period the remainder of the group tolerated the drug until the end of the experiment. On the fifth day the mice receiving 0.075 per cent of diaminodiphenylsulfone in the diet showed signs of toxicity manifested by an extreme hyperactivity and "jumpiness." By the tenth day, 5 mice had died and several others were severely debilitated. The dosage was then reduced to 0.05 per cent. Although this dosage was tolerated, many of the animals later showed a loss of whiskers and of other hair around the snout and a scabby dermatitis around the ano-genital region. When they were sacrificed, all of them exhibited very dark spleens. Many of the mice treated with amithiozone also had dark spleens.

It may be seen from table 1 that half of the control group had died by day 48 and that all of the drug regimens except sulfadiazine were effective in preventing death from infection. Significant suppression of gross lung disease was noted in all of the combined drug groups and in the mice treated with isoniazid and with streptomycin. On the other hand, all treatment regimens suppressed the kidney disease; cycloserine and sulfadiazine to a moderate degree, diaminodiphenylsulfone and isoniazid-diaminodiphenyl-

TABLE 1

Results of Experiment I

| Treatment | Number of Mice | Deaths in Days After Infection* | Average Gross Disease† | |
|---|---|---|---|---|
| | | | Lungs | Kidneys |
| Controls | 10 | 28, 31, 34, 42, 48, 69 | 1.9 | 3.4 |
| Streptomycin, 5 mg./day | 10 | *4, 4* | 1.0 | 0.1 |
| Isoniazid, 0.1% | 10 | — | 0.6 | 0.05 |
| Cycloserine, 0.75% | 5 | *9* | 2.6 | 0.9 |
| Amithiozone, 0.25% | 10 | — | 1.9 | 0.15 |
| Diaminodiphenylsulfone, 0.075% for 10 days, then 0.05% | 10 | *9, 10* | 2.0 | 0 |
| Sulfadiazine, 1.0% | 10 | *13*, 28, 36, 62, 69 | 1.8 | 0.9 |
| Isoniazid-diaminodiphenylsulfone‡ | 10 | *9, 11, 12*, 76 | 0.15 | 0 |
| Isoniazid-amithiozone | 10 | — | 0.45 | 0.2 |
| Isoniazid-streptomycin | 10 | — | 0.35 | 0.15 |
| Isoniazid-streptomycin-diaminodiphenylsulfone | 5 | — | 0.4 | 0.1 |

Treatment from day 3 to day 90; survivors killed on day 90.

* Figures in italics indicate deaths related to drug toxicity.

† Based on scale of 0 to 4 in those mice that died or were killed after day 28.

‡ Dosage of each drug in combined therapy was the same as that used in single-drug regimens.

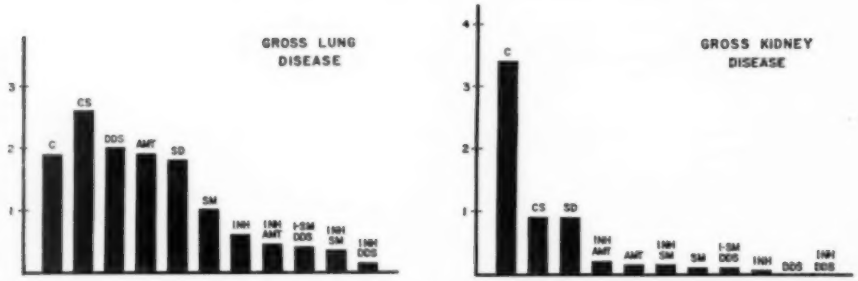


FIG. 1. *Experiment I:* Average amount of disease in lungs and kidneys, based on score of 0 to 4. C—controls; CS—cycloserine; DDS—diaminodiphenylsulfone; AMT—amithiozone; SD—sulfadiazine; SM—streptomycin; INH or I—isoniazid.

sulfone virtually completely. These relationships are shown graphically in figure 1.

Grossly, the lung lesions of untreated mice consisted of scattered small, round, gray foci which were occasionally coalescent. The liver and spleen were usually normal in appearance, except for enlargement of the latter. The kidneys were most striking in their appearance, harboring many large, yellow, protruberant abscesses filled with purulent material. In the more advanced stages both kidneys were almost entirely destroyed by these abscesses. The very early kidney lesions were tiny irregular gray foci. Impression smears made from all of these organs revealed numerous acid-fast bacilli.

Microscopically, the untreated lung lesions consisted mainly of poorly defined dense collections of small round cells producing a diffuse interstitial pneumonitis. Necrosis was minimal. In certain areas the alveoli were filled with large "foamy" macrophages containing acid-fast bacilli. The kidney lesions varied from huge necrotic abscesses replacing almost the entire parenchyma to smaller foci of cellular infiltration. Giant cells, caseation, and fibrosis were not evident in any of the organs.

Under the influence of therapy, the lung lesions tended to be smaller in number and in size, and to consist predominantly of foci of foamy macrophages. Some of the kidneys were grossly scarred and pockmarked, due to replacement of parenchyma by dense mononuclear cell infiltrates. The kidneys which were called "negative" on gross inspection were either devoid of microscopic lesions or contained only small areas of cellular infiltration, sometimes extending inward linearly from the capsule. In one section a large area was seen in which the tissue was replaced by a dense infiltration of macrophages and lymphocytes within which were small cystlike spaces filled with amorphous eosinophilic material, the whole giving an appearance resembling thyroid. In addition to these chronic lesions, one kidney from a mouse given isoniazid-amithiozone showed an acute pyelonephritis with neutrophilic reaction in the epithelial lining and purulent material in the pelvis. Some characteristic sections are depicted in figure 2.

*Comment:* In comparison with the preliminary trial, a greater contrast was evident between treated and control groups in the more virulent and more promptly treated infection of *Experiment I*. All of the drugs except sulfadiazine were effective in reducing mortality, and all but cycloserine and sulfadiazine markedly suppressed kidney disease. The differences in gross lung disease between control and treated mice were not so dramatic. Although combined drug therapy was superior to single-drug therapy in the suppression of lung disease, this was not the case with disease of the kidney.

The next experiment was designed to accentuate differences between drug regimens by providing a still more virulent infection, a longer delay in the start of therapy, and a treatment-free period before the mice were sacrificed.

*Experiment II:* The culture used for infection was obtained directly from the slants inoculated with homogenized lung tissue from a control mouse of *Experiment I*. It had had two mouse passages. The viable count was found to be 30 million units per mouse. Therapy was begun on day 13 and continued until day 52, a total of thirty-nine days, and all survivors were killed on day 70. The following eleven drug regimens were ad-

FIG. 2. Photomicrographs of lung and kidney lesions in mice of *Experiment I*.

*A*. Kidney of untreated mouse that died on day 69, showing replacement of parenchyma by multiple abscesses. (× 15) *B*. Kidney of mouse treated with isoniazid-amithiozone and killed on day 90, showing acute pyelitis and several contracted areas of dense cellular infiltration. (× 15) *C*. Kidney of mouse treated with amithiozone and killed on day 90, showing dense cellular infiltration. (× 50)

*D*. Lung of untreated mouse killed on day 90, showing poorly defined cellular infiltrations without necrosis. (× 43) *E*. Lung of isoniazid-treated mouse killed on day 90, showing subpleural focus of large macrophages and lymphocytes. (× 300) *F*. Same as *E*, showing a similar focus in another area of lung. (× 80)

TABLE 2
RESULTS OF EXPERIMENT II

| Treatment | Number of Mice | Deaths in Days After Infection* | Average Gross Disease† | |
|---|---|---|---|---|
| | | | Lungs | Kidneys |
| Controls | 11 | 13, 24, 27, 28, 31, 31, 32, 35, 45, 45, 52 | 2.8 | 3.3 |
| Cycloserine, 0.75% for 16 days then 0.56% | 9 | *15, 20, 20, 21, 22*, 63 | 1.5 | 2.9 |
| Amithiozone, 0.25% | 10 | *26*, 47, 52, 53, 56, 60, 69 | 2.2 | 2.8 |
| Diaminodiphenylsulfone, 0.04% | 9 | 27, 59 | 1.4 | 2.6 |
| Streptomycin 5 mg./day for 7 days then 2.5 mg./day | 10 | *19* | 1.7 | 0.8 |
| Isoniazid, 0.1% | 10 | 17, 55 | 1.3 | 0.9 |
| Isoniazid-sulfadiazine, 0.1% and 0.75% for 16 days, then 0.075% and 0.56% | 8 | *20, 22, 23, 23, 23* | 2.0 | 1.5 |
| Isoniazid-cycloserine, 0.1% and 0.75% for 16 days, then 0.075% and 0.56% | 8 | *16, 17, 25*, 49 | 2.0 | 1.2 |
| Isoniazid-diaminodiphenylsulfone‡ | 9 | *18, 24* | 1.5 | 0.8 |
| Isoniazid-amithiozone | 10 | 40 | 1.2 | 0.3 |
| Isoniazid-streptomycin | 10 | *15, 15* | 1.5 | 0.25 |
| Isoniazid-streptomycin-diaminodiphenylsulfone | 9 | *14, 15, 16*, 48, 49 | 1.3 | 0.2 |

Treatment from day 13 to day 52; survivors killed on day 70.
* Figures in italics indicate deaths related to drug toxicity.
† Based on scale of 0 to 4, in those mice that died or were killed after day 28.
‡ Dosage of each drug in combined therapy was the same as that used in single-drug regimens.

ministered: streptomycin, isoniazid, cycloserine, amithiozone, diaminodiphenylsulfone, isoniazid-sulfadiazine, isoniazid-cycloserine, isoniazid-diaminodiphenylsulfone, isoniazid-amithiozone, isoniazid-streptomycin, and isoniazid-streptomycin-diaminodiphenylsulfone. (*See* table 2 for dosages.)

Cultures of the urine were made on several occasions after treatment had been discontinued. Three to 5 mice were placed in a metabolism cage, and the urine was collected overnight in a sterile flask. After digestion with sodium hydroxide, neutralization, and one washing with distilled water, the sediment was planted on solid medium slants or plates. Sections of the brain were cut after the entire head had been decalcified and fixed in Hanke's solution for two or three days.

Premature deaths from drug toxicity necessitated a reduction in the dosage of three drugs: cycloserine, sulfadiazine, and streptomycin. After sixteen days of therapy the concentration of cycloserine and of isoniazid-cycloserine was reduced by diluting 3 parts of drug food with 1 part of untreated food. The same procedure was followed for the isoniazid-sulfadiazine medication. Episodes of collapse with labored breathing, similar to those noted in *Experiment I*, were observed in many of the mice immediately following subcutaneous injections of 5 mg. of streptomycin. After six deaths had occurred the dosage was reduced to 2.5 mg. per day. Side effects of diaminodiphenylsulfone administration (spotty loss of hair, ulcerative lesions of the skin in the anogenital region, and dark spleens) were again noted. In addition, toxicity from diaminodiphenylsulfone undoubtedly contributed to the early deaths in each of the three groups receiving the drug, since these mice had relatively little gross disease.

The data concerning mortality and gross disease are presented in table 2 and figure 3. All of the untreated mice had died by the fifty-second day, and again each treatment regimen was effective in preventing deaths from infection during therapy. The amithiozone-treated mice, however, succumbed rapidly as soon as therapy was discontinued. Extensive lesions similar to those described in the previous experiment were observed in most of the untreated mice. Reduction in the amount of gross lung disease was not dramatic with any regimen, and roughly equivalent scores were achieved with streptomycin, cycloserine, diaminodiphenylsulfone, isoniazid, and most of the combined regimens. The addition of sulfadiazine and of cycloserine to isoniazid produced results which were slightly inferior to those ob-

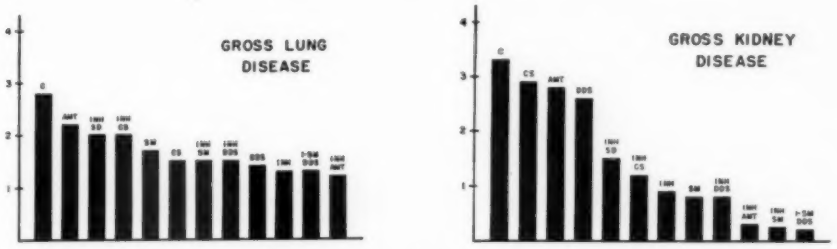


FIG. 3. *Experiment II:* Average amount of gross disease in lungs and kidneys, based on score of 0 to 4. (*See* Figure 1 for abbreviations).

served with isoniazid alone, probably due to toxic effects produced by the two former drugs. Amithiozone was the least effective therapy.

An entirely different picture was presented by comparison of the kidney scores. Here there was a sharp contrast between the drugs that had little or no effect (cycloserine, amithiozone, diaminodiphenylsulfone) and the highly effective combined-drug regimens (isoniazid-amithiozone, isoniazid-streptomycin, and triple drugs). The results with isoniazid alone, streptomycin alone, and isoniazid-diaminodiphenylsulfone were only slightly inferior to those obtained with the highly effective regimens. Again, the addition of sulfadiazine or of cycloserine diminished slightly the activity of isoniazid.

Attempts to isolate photochromogenic mycobacteria from pooled urines on days 56 and 62 were successful in groups treated with cycloserine, diaminodiphenylsulfone, isoniazid-cycloserine, and isoniazid-amithiozone. Urines from other groups yielded negative cultures.

A curious syndrome known as "spinning disease" was encountered in this experiment. Affected mice became ataxic, held their heads to one side and, when held up by their tails, whirled around with enough force to raise themselves to a horizontal plane. After such a spinning exhibition the animals often walked around in circles for a few minutes. The syndrome was observed first on day 17 in 2 mice in the triple-drug group, then in one or 2 mice treated with isoniazid-streptomycin and with streptomycin alone during the next ten days. One untreated mouse became a spinner on day 24 and continued to spin until it died eight days later. The syndrome also became evident in one amithiozone-treated mouse on day 41, and on day 62 in one diaminodiphenylsulfone mouse and in 2 isoniazid-diaminodiphenylsulfone mice. Although the spinning disease usually persisted once it began, most of the affected mice in the treated groups continued to appear otherwise healthy.

Microscopic sections of the brains of 4 untreated mice revealed numerous abscesses of various sizes. These lesions were sharply demarcated from the surrounding parenchyma, contained degenerating nuclei and other necrotic material in their centers, and showed peripheral infiltrations of small round cells. There was no fibrous encapsulation. The meninges and the ventricular system were not involved in the areas examined, although a few of the abscesses were located in close proximity to them. In 3 instances, small caseous granulomas containing many acid-fast bacilli were seen in the bones of the skull. Representative brain and bone sections are presented in figure 4.

There was only one "spinner" among the untreated mice and, unfortunately, sections through the inner ear of this animal were not obtained. Sections of the cochlea and semicircular canals of two "nonspinners" revealed no lesions, although large abscesses were present in the brain of one.

Acid-fast bacilli were visible in most of the brain lesions and, in addition, they were found within cells in some normal-appearing areas of bone marrow at the base of the skull.

*Comment:* Although the number of viable units in the inoculum was approximately the same as in the previous experiment, the infection of *Experiment II* was more severe. The greater severity is attributed to the fact that the strain had had an additional mouse passage. The presence of viable bacilli in the urine after therapy and of acid-fast bacilli in impression smears and histologic sections from many organs (including

the brain and bone marrow), as well as the observation of signs of inner ear involvement, emphasized the widespread distribution and multiplication of photochromogenic mycobacteria in the tissues of the mouse. The next experiment was designed to determine more exactly the fate of bacilli in various organs and the influence of chemotherapy upon their multiplication.

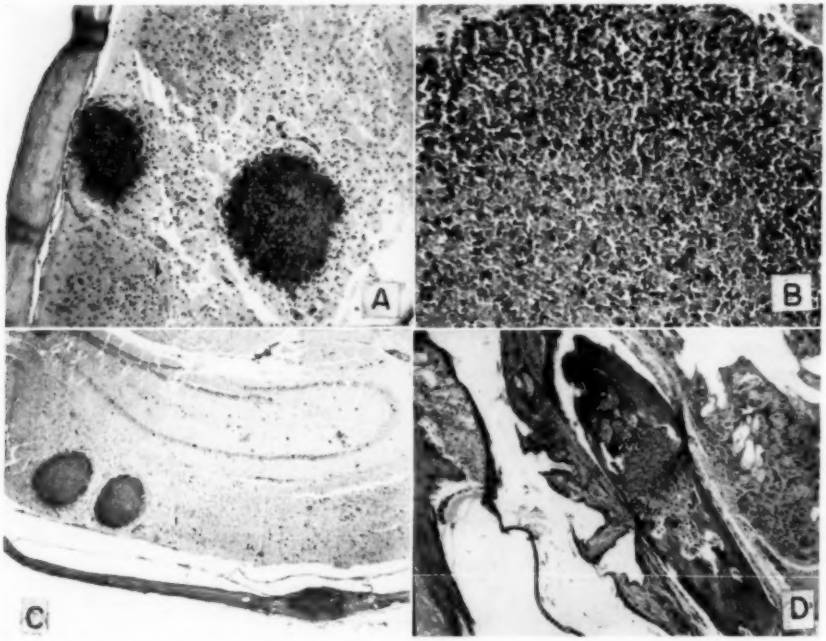


FIG. 4. Brain and skull lesions in untreated mice of *Experiment II*.
*A*. Two abscesses in brain of mouse that died on day 32. Note lack of meningeal reaction. (× 125) *B*. Higher power of edge of brain abscess. (× 200)
*C*. Multiple brain abscesses and granulomatous lesion in bone producing spindle-shape enlargement of skull. (× 60) *D*. Granulomatous focus in bone next to middle ear. (× 125)

TABLE 3
EXPERIMENT III. AVERAGE AMOUNT OF GROSS DISEASE IN LUNGS AND KIDNEYS BASED ON SCALE OF 0 TO 4*

| Treatment | Days of Infection/Days of Treatment | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/0 | | 13/0 | | 21/6 | | 32/17 | | 49/35 | | 73/55 | |
| | Lungs | Kidneys | Lungs | Kidneys | Lungs | Kidneys | Lungs | Kidneys | Lungs | Kidneys | Lungs | Kidneys |
| None | 0 | 0 | 1.5 | 0.4 | 2.2 | 1.7 | 2.4 | 3.5 | 2.2 | 3.5 | — | — |
| SV, 0.1% | | | | | | | | | 2.7 | 3.8 | — | — |
| DDS, 0.04% | | | | | 1.5 | 2.0 | 2.5 | 3.1 | 1.5 | 2.7 | 1.3 | 3.2 |
| SM, 2.5 mg./day | | | | | 1.7 | 0.2 | 2.0 | 1.7 | 1.5 | 1.2 | 1.5 | 0.7 |
| INH, 0.1% | | | | | 1.7 | 1.0 | 1.9 | 2.1 | 1.7 | 1.7 | 1.5 | 2.0 |
| SM-INH | | | | | 2.0 | 0.6 | 2.0 | 0.8 | 1.2 | 0.8 | 1.2 | 0.5 |

SV = streptovaricin, DDS = diaminodiphenylsulfone, SM = streptomycin, INH = isoniazid
* Average of 3 to 5 mice in each group

*Experiment III:* The inoculum consisted of 3 million viable units of the photochromogenic mycobacteria which had had three mouse passages. Treatment was started on day 15 and continued through day 70, a total of fifty-five days. Survivors were killed on day 73. Five drug regimens were evaluated, namely, isoniazid, streptomycin, diaminodiphenylsulfone, streptovaricin, and isoniazid-streptomycin. Urine was collected overnight at frequent intervals from groups of 4 to 6 mice, as well as from individual mice in a few instances. Cultures were made from the urine as outlined in *Experiment II*. Several methods were used to culture the heart's blood, until the following technique was found to be the most satisfactory: As much blood as possible was withdrawn aseptically and immediately mixed with 20 volumes of sterile water. After refrigeration overnight, the laked blood was centrifuged and the sediment was planted on slants of solid medium.

At the time periods shown in table 3, mice were killed with chloroform and examined at autopsy immediately. Enumeration of viable bacilli was accomplished as follows: the brain, the left kidney, the spleen, approximately one-fifth of the liver, and both lungs were removed aseptically and frozen at −30° C. for two to six weeks. After thawing, each organ was homogenized with a mortar and pestle, and the homogenate was brought to a volume of 1 ml. with sterile physiologic saline. Appropriate tenfold dilutions in saline were dropped in triplicate in 0.02 ml. amounts on plates of Middlebrook oleic-acid-albumin agar. Through day 31 each average viable count was calculated from a group of 4 mice; on day 49 from a group of 3; and at the end of the experiment from 4 mice in the isoniazid-streptomycin group, 3 in the diaminodiphenylsulfone group, and 2 each in the isoniazid and the streptomycin groups. It will be noted that at the end of the experiment cultures of blood and tissue were obtained three days after therapy had been discontinued.

Drug toxicity was not a problem in this experiment, although dark spleens were observed in the diaminodiphenylsulfone-treated mice after thirty-five days of treatment. Notwithstanding the tenfold smaller inoculum, the infection was as severe as the preceding one. The untreated mice began to succumb on day 14, and their lungs and kidneys contained lesions similar in extent to those seen in *Experiment II*. In the control group, 19 mice were sacrificed at predetermined intervals, 7 died spontaneously of their disease between days 14 and 55, and only one remained alive at the end of the experiment. In the treated groups there were only one or two spontaneous deaths; the remainder of the mice were killed for sampling. The drug regimens and a summary of the gross-lesion scores are presented in table 3, from which one may follow the progress of the disease in each group. In the untreated group, the amount of disease in the lungs increased until day 20, then remained constant during the remainder of the experiment. The kidney lesions increased in severity until day 31, at which time there was

TABLE 4

RECOVERY OF MYCOBACTERIA FROM THE URINE

| Days of Infection | Days of Treatment | Drug Regimen: Untreated | Streptovaricin | DDS* | Isoniazid | Streptomycin | Isoniazid-Streptomycin |
|---|---|---|---|---|---|---|---|
| 1 | 0 | − | | | | | |
| 3 | 0 | − | | | | | |
| 8 | 0 | + | | | | | |
| 16 | 0 | + | | | | | |
| 22 | 7 | + | + | + | − | + | + |
| 29 | 14 | Contaminated culture | | + | − | + | − |
| 45 | 30 | + | + | | | − | |
| 56 | 41 | + | + | | | − | |
| 62 | 47 | | | | Contaminated culture | | − |

* Diaminodiphenylsulfone.

\+ Indicates growth of photochromogenic mycobacteria.

− Indicates negative culture.

Blank space indicates culture not done.

TABLE 5

RECOVERY OF MYCOBACTERIA FROM HEART'S BLOOD

| Days of Infection | Days of Treatment | Drug Regimen: Untreated | DDS* | Isoniazid | Streptomycin | Isoniazid-Streptomycin |
|---|---|---|---|---|---|---|
| 1 | 0 | 2/2† | — | — | — | — |
| 14 | 0 | 4/4 | — | — | — | — |
| 21 | 6 | 3/3 | 3/3 | 2/3‡ | 2/3 | 3/3 |
| 32 | 17 | 3/3 | 4/4 | 4/4 | 2/4 | 3/4 |
| 50 | 35 | 3/3 | 3/3 | 2/3 | 1/3 | 2/3 |
| 73 | 55§ | 1/1 | 3/3 | 2/2 | 2/2 | 3/3 |

* Diaminodiphenylsulfone

† Number of mice with positive blood culture over number of mice cultured

‡ One culture contaminated

§ Three days following termination of treatment

almost total involvement of the organ. All of the drugs except streptovaricin retarded the development of gross disease. As in the previous experiment, the most striking evidence of drug effect was noted in the kidney, and diaminodiphenylsulfone was less effective then streptomycin or isoniazid.

The results of the urine cultures are shown in

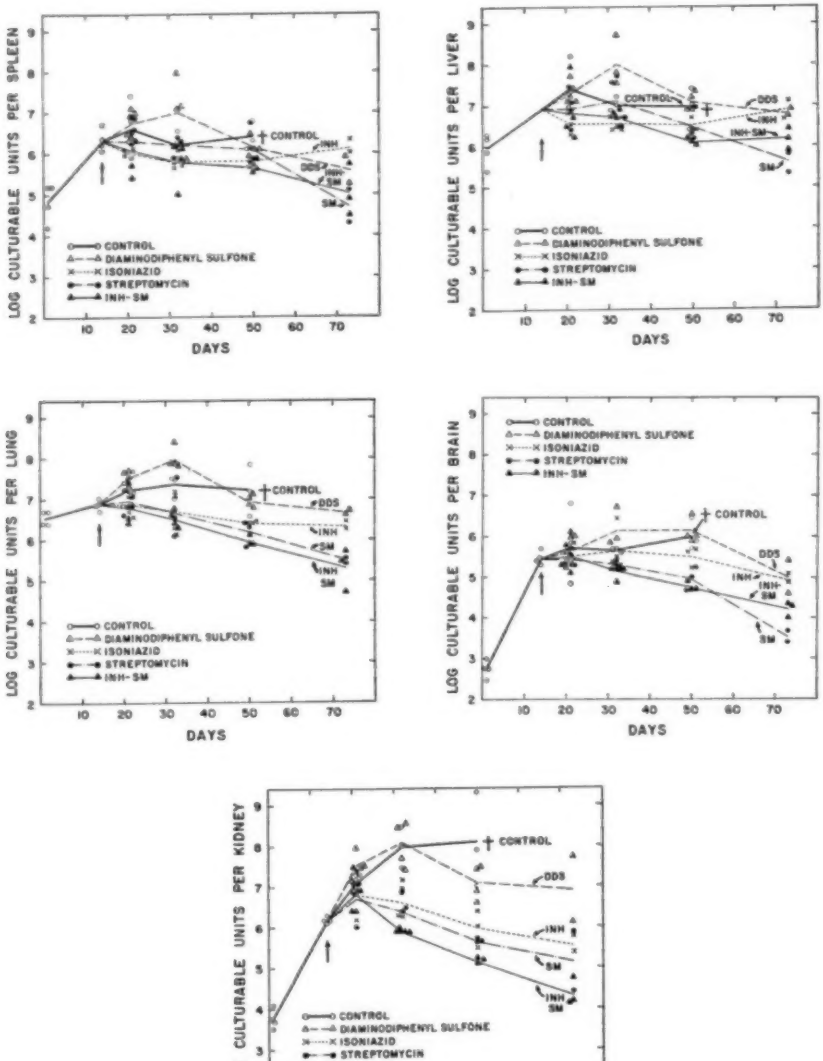


FIG. 5. Average number of viable bacilli in various organs of control and treated mice of *Experiment III*. Arrow indicates start of therapy. (Liver counts were omitted at 14 days.)

table 4. Mycobacteria were recovered consistently from untreated mice from the eighth day onward. Treatment with streptovaricin and with diaminodiphenylsulfone did not render the cultures negative, whereas isoniazid, streptomycin, and isoniazid-streptomycin were effective in sterilizing the urine of mycobacteria.

The data on blood cultures are contained in table 5. It may be seen that throughout the experiment mycobacteria were recovered consistently from the heart's blood regardless of therapy.

The results of enumeration of viable bacilli in the various organs are presented in figure 5. The mycobacteria localized very rapidly in the lungs, increasing in number but little thereafter. In contrast, there were relatively few bacilli in the kidney and in the brain twenty-four hours after infection, but a large increase had occurred by the fourteenth day. Thereafter the brain count remained stable while the bacilli in the kidney continued to increase in number until the thirty-second day. In the livers and spleens the counts increased for twenty-one days, and the census then stabilized. The effect of chemotherapy on the bacterial count was slight in the spleen and in the liver, more obvious in the lungs and in the brain, and most dramatic in the kidney. Even in the latter organ, however, the count diminished slowly, and there were still more than ten thousand viable units per kidney after fifty-five days of treatment with the most favorable regimen. Diaminodiphenylsulfone was obviously inferior to the other drug regimens, and although eventually depressed, each diaminodiphenylsulfone curve rose above the control curve at twenty-one to thirty-two days. Streptomycin and isoniazid-streptomycin seemed to be slightly more effective than isoniazid, but this was not clear-cut. Streptovaricin-treated mice were not included in the enumeration effort.

Only 2 mice in this experiment exhibited signs of spinning disease, one treated with streptomycin and one with isoniazid-streptomycin. They were sacrificed at the end of the experiment, and neither had a higher count of bacilli in the brain than their respective nonspinning mates. Several cultures recovered from mice treated for thirty-five days were tested for susceptibility to isoniazid and streptomycin. None of them showed an increase in resistance.

*Comments:* These findings prove the widespread distribution and multiplication of photochromogenic mycobacteria throughout the body of the mouse and the relatively feeble antimicrobial powers of presently available drugs against this strain. Better effects in the kidney are probably related to higher drug concentrations afforded by excretion through this organ. Because of its poor penetration into the brain of man, the ability of streptomycin to lower the count in the brain even more effectively than isoniazid was a surprise.

## Discussion

On the basis of these experiments, it is now possible to list many drug regimens in their order of effectiveness in a standarized mouse infection produced by a single strain of photochromogenic mycobacteria. For single drugs, the most effective are isoniazid and streptomycin; less beneficial are amithiozone and diaminodiphenylsulfone; only very slightly effective are cycloserine and sulfadiazine. Streptovaricin is apparently ineffective. In combination with isoniazid, neither cycloserine, diaminodiphenylsulfone, amithiozone, nor sulfadiazine is additive or synergistic. In certain instances cycloserine and sulfadiazine each may be slightly antagonistic to the action of isoniazid. The combination of isoniazid and streptomycin is perhaps slightly more beneficial than either drug alone.

Para-aminosalicylic acid was not evaluated in these studies because of its uniformly poor inhibitory effect *in vitro* against atypical mycobacteria and its relatively weak antituberculous power against mouse infections produced by the more susceptible *M. tuberculosis*. In view of cycloserine's ability to influence ordinary tuberculosis in man despite its failure to do so in the mouse, this drug deserves clinical trial in atypical mycobacterial infections notwithstanding its poor performance in the experiments reported herein. Another compound which deserves further experimental, and possible clinical, trial is thiocarbanilide and related substances (4, 6).

The true significance of the limited success of chemotherapy in these experiments may only be judged with the knowledge that *in vitro* the photochromogenic strains tend to be the most drug susceptible of all of the atypical mycobacteria, especially to isoniazid. Thus, clinical infections associated with scotochromogenic, nonchromogenic, and rapidly growing mycobacteria may reasonably be expected to respond even less

well. Chemotherapy experimentation with infections produced by atypical mycobacteria other than photochromogens is handicapped by lack of a susceptible animal host, except in the case of *M. fortuitum*. One approach would be the utilization of silicotic guinea pigs, whose susceptibility to several atypical mycobacterial strains has recently been demonstrated ( 9, 10 ).

In man, the pulmonary lesions associated with photochromogenic mycobacteria are very similar to those seen in ordinary tuberculosis. In mice, however, these bacilli produce lesions not ordinarily seen when *M. tuberculosis* is the infecting organism. The outstanding feature after intravenous infection is a progressive kidney disease, characterized by abscesses and dense foci of cellular infiltration. Similar lesions have been observed when mice have been injected with *M. fortuitum* (11–13) or with *M. smegmatis* (14). In addition, abscesses in the brain may be produced with photochromogenic mycobacteria, as with *M. smegmatis* (14).

Spinning disease of mice is not uncommon, having been described following intravenous infection with *Pseudomonas pyocyanea* (15), with a species of Nocardia (16), and with other mycobacteria (11, 12, 17). Gorrill (16) and Penso (11) describe specific lesions in the cochlea and/or the semicircular canals to account for the spinning.


## Summary

A number of drug regimens were compared in their ability to suppress infection produced by a single strain of photochromogenic mycobacteria in mice. The criteria for effectiveness included survival time, amount of gross disease, histology of lesions, serial enumeration of viable bacilli in five organs, and the presence of viable bacilli in the urine and the heart's blood. The most effective single drugs were isoniazid and streptomycin. Diaminodiphenylsulfone and amithiozone demonstrated slight suppression, while cycloserine and sulfadiazine were only questionably beneficial. Streptovaricin was ineffective. The combination of isoniazid and streptomycin was only slightly more effective than either drug alone, and none of the other agents increased the effectiveness of isoniazid when given in combination with that drug.

Therapy with isoniazid, streptomycin, and the combination of both succeeded in sterilizing the urine of mycobacteria; treatment with streptovaricin and diaminodiphenylsulfone did not. Bacilli were recovered consistently from heart's blood cultures for seventy-three days regardless of treatment. Chemotherapy exerted its greatest effect on the kidney disease, had less effect on the mycobacteria in the lungs and brain, and had but little influence on the bacterial count in the spleen and liver.

The pathogenicity of this strain of photochromogenic mycobacteria was enhanced by serial passage through mice. When injected intravenously, the bacilli were capable of widespread localization and multiplication in the organs of the mouse, thereby producing progressive abscesses in the kidneys, focal and diffuse cellular infiltrates in the lungs, scattered brain abscesses, granulomatous bone lesions, and "spinning disease."



## Sumario

### *La Quimioterapia y Anatomía Patológica de las Infecciones Micobacterianas Fotocromógenas Experimentales*

Se compararon varios regímenes farmacológicos con respecto a su capacidad para suprimir la infección producida por una sola cepa de micobacterias fotocromógenas en los ratones. Las pautas de la eficacia comprendieron tiempo de sobrevivencia, proporción de enfermedad macroscópica, histología de las lesiones, enumeración seriada de los bacilos viables en cinco órganos y presencia de bacilos viables en la orina y en la sangre del corazón. Las drogas más eficaces por sí solas fueron la isoniacida y la estreptomicina. La diaminodifenilsulfona y la amitiozona produjeron leve supresión mientras que la cicloserina y la sulfadiacina no fueron más que dudosamente beneficiosas. La estreptovaricina resultó ineficaz. La combinación de isoniacida y estreptomicina fué apenas más eficaz que una u otra droga por sí sola y ninguno de los otros medicamentos usados acrecentó la eficacia de la isoniacida cuando se administró en combinación con dicha droga.

La terapéutica con isoniacida, estreptomicina y la combinación de ambas logró esterilizar la orina con respecto a micobacterias; no lo hizo el tratamiento con estreptovaricina y diaminodifenilsulfona. Independientemente del tratamiento, se obtuvieron bacilos constantemente de los cultivos de la sangre del corazón por espacio de setenta y tres días. La quimioterapia ejerció su mayor efecto sobre la afección renal, mostró menos efecto sobre las micobacterias de los pulmones y del cerebro y manifestó muy poco

influjo sobre la fórmula bacteriana en el bazo y el hígado.

La patogenicidad de esta cepa de micobacterías fotocromógenas fué realzada por el pase seriado a través de ratones. Al ser inyectados intravenosamente, los bacilos se mostraron capaces de localización difusa y proliferación en los órganos del ratón, produciendo de ese modo abscesos evolutivos en los riñones, infiltraciones celulares locales y difusas en los pulmones, abscesos cerebrales esparcidos, lesiones óseas granulomatosas y la llamada "enfermedad de hebras."


## Resume

*Chimiothérapie et pathologie des infections mycobactériennes photochromogéniques*

Un certain nombre de régimes medicamenteux ont été comparés à l'égard de leur capacité de suppression de l'infection produite chez la souris par une seule souche de mycobactéries photochromogènes. Le critère d'efficacité comprenait le temps de survie, le nombre des lésions macroscopiques, l'histologie des lésions, l'énumération sérielle de bacilles viables dans cinq organes et la présence de bacilles viables dans l'urine et le sang cardiaque. Administrées seules, les drogues les plus efficaces furent l'isoniazide et la streptomycine. La diaminodiphénylsulfone et l'amithiozone n'ont montré qu'un léger effet suppressif, tandis que l'efficacité de la cyclosérine et de la sulfadiazine paraissait douteuse. La streptovaricine était inefficace. La traitement combiné isoniazide-streptomycine n'a été que légèrement plus efficace que l'administration séparée de l'une ou l'autre de ces drogues. Aucun des autres agents associés a l'isoniazide n'augmentait l'efficacité de celle-ci.

Le traitement avec l'isoniazide, la streptomycine et l'association de ces deux drogues faisait disparaître les mycobactéries de l'urine; le traitement avec la streptovaricine et la diaminodiphenylsulfone n'y parvenait pas. Les bacilles étaient constamment isolés dans les cultures de sang cardiaque pendant soixante-treize jours quel qu'ait été le traitement. La chimiothérapie exerçait son effet maximum sur la maladie rénale, un effet moindre sur les mycobactéries du poumon et du cerveau et influençait peu la numération bactérienne dans la rate et le foie.

La pathogénicité de cette souche de mycobactéries photochromogènes était amplifiée par le passage en série sur les souris. Les bacilles inoculés par voie veineuse avaient la capacité de se localiser et de se multiplier dans les organes des souris en produisant des abcès répétés dans les reins et des infiltrats cellulaires focaux ou diffus dans les poumons, des abcès cérébraux disséminés, des lésions osseuses granulomateuses et ce que l'on dénomme "spinning disease."



*Acknowledgments*

The excellent technical assistance of Miss Jeanne Fell is gratefully acknowledged. Drugs were generously supplied as follows: isoniazid and streptomycin by Chas. Pfizer & Co.; diaminodiphenylsulfone and sulfadiazine by Parke, Davis & Co.; hinconstarch and amithiozone by Nepera Chemical Co.; cycloserine by Eli Lilly & Co.; and streptovaricin by The Upjohn Co.


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# BIOCHEMICAL ANALYSIS OF THE VIRULENCE OF TUBERCLE BACILLI[1]

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## INTRODUCTION

It was reported by the present writers in previous papers (1, 2) that cord factor, a toxic glycolipid extracted from *Mycobacterium tuberculosis* by Bloch and associates (3, 4), exerts an inhibiting effect on the succinic dehydrogenase system and some diphosphopyridine nucleotide-linked (lactic, malic and α-glycerophosphoric) dehydrogenase of the liver of mice. This effect on the respiratory metabolism of host tissues was considered to be in part the basic mechanism of the toxicity of cord factor, which has been repeatedly described by Bloch and his collaborators (3, 5, 6) and was recently confirmed by the present writers (1).

The mechanism of this inhibition reaction was studied in some detail, and it was assumed from the available data that the metabolic lesion caused by the injection of cord factor is a quantitative decrease of an unknown soluble factor or factors which accelerate the activity of the succinic dehydrogenase of the mouse liver mitochondria (2). This unknown factor is now under investigation.

In order to correlate the above mentioned biochemical activity of cord factor with the virulence of tubercle bacilli, an enzymatic study of the tuberculous infection of mice was attempted. The present paper deals with the metabolic alteration of the infected tissues in experimental murine tuberculosis, and the significance of the role of cord factor in the virulence of tubercle bacilli was verified.

## MATERIALS AND METHODS

*Animals:* A pure line of Swiss Albino mice, strain Na-2, was obtained from a single source. Since the enzymic activity considerably fluctuated according to the age or sex of mice used, male animals at the fourth to sixth weeks of age, weighing 10 to 15 gm., were used. They were fed pellet diet[2] and water *ad libitum*. One experimental group consisted of at least 5 animals, as described in the foregoing papers (1, 2).

*Bacterial strains:* The following strains of tubercle bacilli and the saprophytic mycobacteria were used together with some other bacterial strains which are listed below: Ravenel, highly virulent bovine tubercle bacilli; H37Rv, virulent human tubercle bacilli; BCG, attenuated bovine tubercle bacilli; H37Ra, avirulent human tubercle bacilli; *Mycobacterium phlei*, *Mycobacterium smegmatis*, *Mycobacterium 607*, and *Mycobacterium butyricum*, saprophytic mycobacteria.

The first seven strains were obtained in 1954–1956 through the courtesy of Dr. Ken Yanagisawa, Division of Tuberculosis, National Institute of Health, Tokyo. *Mycobacterium butyricum* was obtained in 1956 through the courtesy of Dr. Jules Freund, Division of Applied Immunology, Public Health Research Institute of the City of New York, New York.

Full growth of these strains in Sauton synthetic liquid medium was used for the preparation of the bacterial suspension.

The bacterial strains used in the present study other than genus mycobacteria are as follows: *Salmonella typhi* (strain H901 W); *Micrococcus pyogenes* var. *aureus* (strain 209 P); *Brucella melitensis* (strain No. 4); *Shigella dysenteriae* (strain Shiga); *Bacillus anthracis* (strain 1 Byo).

All five strains were obtained through the courtesy of Dr. Tsunesaburo Fujino, Research Institute for Microbial Diseases, Osaka University, Osaka.

For the preparation of bacterial suspension, these strains were grown for twenty-four to forty-eight hours on agar slants, except for *Brucella melitensis* which was grown for forty-eight hours on blood agar.

Besides these bacterial strains, Ehrlich's ascites tumor was used in some experiments, the stock inoculum of which was obtained through the courtesy of Dr. Juntaro Kamahora, Research Institute for Microbial Diseases, Osaka University, Osaka.

*Assay of the succinic dehydrogenase activity of mouse liver:* As previously described (1), the succinic dehydrogenase activity of the homogenate of liver of mice was determined by Kun and Aboad's method (7) and the enzymic activity was expressed as $\gamma$ of formazan reduced per mg. $N$ of liver homogenate per minute plus-minus standard deviation. The significance of the difference of the values between the control and the experimental groups was statistically examined.

[1] From the Research Laboratory of Toneyama Hospital, National Sanatorium, Osaka; and the Department of Biochemistry, Kyushu University School of Medicine, Fukuoka, Japan.

[2] Oriental Pellet Diet MC 5, Oriental Yeast Co., Osaka, Japan.

*Nitrogen determinations:* Nitrogen of the liver homogenate was determined by the micro-Kjeldahl method.

*Assay of the catalase activity of mouse liver:* The catalase activity of the liver of mice was determined by the modified method of Euler and Josephson (8), and the catalase activity was expressed as Kat. F plus-minus standard deviation.

## Results

*The effect of the intravenous injection of heat-killed bacilli of various mycobacterial strains on the succinic dehydrogenase activity of mouse liver:* It is shown in table 1 that the activity of the succinic dehydrogenase system of the mouse liver is markedly decreased by the intravenous injection of 0.3 mg. dry weight of the heat-killed virulent bovine (Ravenel) and 1.0 mg. dry weight of human (H37Rv) tubercle bacilli. The same enzymic activity, however, is not affected by the intravenous injection of 1.0 mg. dry weight of either attenuated bovine (BCG) or avirulent human (H37Ra) tubercle bacilli. Four strains of the nonpathogenic mycobacteria (*M. phlei*, *M. smegmatis*, *M. 607*, and *M. butyricum*) are also without effect on the succinic dehydrogenase activity of the mouse liver. Thus, the phenomenon seems to be specific for the virulent strains of *Mycobacterium tuberculosis* among the different strains of mycobacteria tested.

*The change of the succinic dehydrogenase activity of the mouse liver during the course of living infection with various strains of tubercle bacilli:* As shown in figure 1, the per cent of activity of the succinic dehydrogenase of the mouse liver was rapidly decreased during the course of living Ravenel infection. The strain Ravenel is highly virulent for mice, as reported by McKee and associates (9) and by the present writers (10). It was reported that the intravenous infection of mice with 200,000 viable counts killed mice within twenty-two days (9) and with 256,000 viable counts, within two weeks (10). The viable counts present in the whole body per mouse were determined by the homogenate technique of whole body of mice (11) in parallel with the assay of the succinic dehydrogenase activity of the liver. The data of both determinations are summarized in table 2. It is evident from these data that the activity of the succinic dehydrogenase of the mouse liver is decreased parallel with the increase of viable counts in whole body, *i.e.*, the development and establishment of the fatal virulent infection.

Strain H37Rv is less virulent for mice than

TABLE 1

Effect of Heat-killed Bacilli of Different Strains of Mycobacteria on Succinic Dehydrogenase Activity of Mouse Liver

| Virulence of Strain | Strain Injected | Dry Weight of Inoculum | γ of Formazan Reduced per Mg. N per Min. (Mean ± Standard Deviation) |
|---|---|---|---|
| | | *mg.* | |
| Virulent | Ravenel | 0.3 | 1.16 ± 0.78 Significant ($\alpha$ = 0.01) |
| | | Saline | 3.44 ± 1.36 |
| | H37Rv | 1.0 | 3.15 ± 0.77 Significant ($\alpha$ = 0.01) |
| | | 0.3 | 4.50 ± 0.60 Not significant |
| | | Saline | 4.72 ± 0.81 |
| Attenuated | BCG | 1.0 | 2.80 ± 0.81 Not significant |
| | | Saline | 3.23 ± 0.41 |
| Avirulent | H37Rv | 1.0 | 4.30 ± 0.38 Not significant |
| | | Saline | 3.54 ± 0.61 |
| Nonpathogenic | *M. phlei* | 1.0 | 2.84 ± 0.51 Not significant |
| | *M. smegmatis* | 1.0 | 2.34 ± 0.42 Not significant |
| | *M. 607* | 1.0 | 2.78 ± 0.59 Not significant |
| | | Saline | 2.56 ± 0.56 |
| | *M. butyricum* | 1.0 | 3.66 ± 0.82 Not significant |
| | | Saline | 3.54 ± 0.61 |

Bacilli were killed by heating at 100° C. for 30 minutes and injected intravenously. The enzymic activity was determined 24 hours after the injection. One group of mice consisted of 5 male animals of the same age.

strain Ravenel (9, 10). In close accord with this fact, it was noted that the succinic dehydrogenase activity of the liver of mice infected with strain H37Rv was decreased at a slower rate. Four weeks after infection, approximately half of the infected mice survived the infection. The succinic dehydrogenase activity in the liver of these survivors reached normal levels again. During the course of BCG infection, too, a slight decrease of the succinic dehydrogenase activity of the liver was noted, but it soon reached its normal activity again.

*Effect of heat-killed cells of different pathogenic microorganisms on the succinic dehydrogenase activity of mouse liver:* The results in table 3 show the effect of the intravenous injection of 0.1 to 1.0 mg. dry weight of heat-killed cells of various pathogenic microorganisms.

It was noted that the heat-killed cells of *Salmonella typhi*, among the different bacterial strains tested, caused a significant decrease of the succinic dehydrogenase activity of the liver of mice twenty-four hours after the intravenous injection of 0.3 mg. dry weight. Thus, the decrease of the succinic dehydrogenase activity is not necessarily specific for the tuberculous infection.

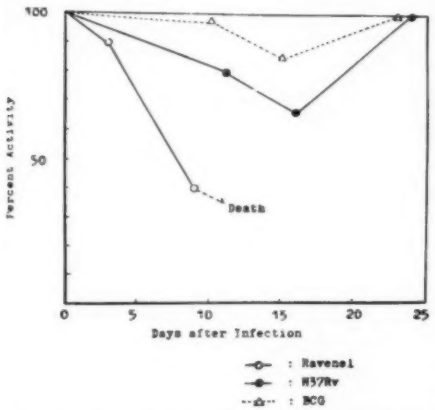


FIG. 1. Decrease of succinic dehydrogenase activity of liver during infection with different strains of *Mycobacterium tuberculosis*. Mice received intravenously 0.01 mg. of living bacilli. Five animals were killed at various intervals of time as well as 5 control animals and the per cent of activity was calculated as follows:

$$\text{Per cent of activity} = \frac{\text{Mean } \gamma \text{ of formazan reduced per mg. N per min. (infected)}}{\text{Mean } \gamma \text{ of formazan reduced per mg. N per min. (noninfected)}}$$

TABLE 2

CORRELATION BETWEEN DECREASES OF SUCCINIC DEHYDROGENASE ACTIVITY OF LIVER AND *IN VIVO* GROWTH OF TUBERCLE BACILLI IN MURINE TUBERCULOSIS

| Days after Infection | Succinic Dehydrogenase in Liver* ($\gamma$ of Formazan Reduced per Mg. N per Min.) | Total Number of Bacilli per Mouse |
|---|---|---|
| 3 | $3.30 \pm 2.11$ Not significant | $3.69 \times 10^4$ |
| | Saline control $3.46 \pm 1.40$ | $5.70 \times 10^4$ |
| | | $1.22 \times 10^5$ |
| | | $1.81 \times 10^5$ |
| | | $4.88 \times 10^4$ |
| 9 | $1.18 \pm 0.44$ Significant ($\alpha = 0.01$) | $1.88 \times 10^6$ |
| | | $2.72 \times 10^5$ |
| | Saline control $2.86 \pm 0.38$ | $1.24 \times 10^6$ |
| | | $1.34 \times 10^5$ |
| | | $1.08 \times 10^6$ |

$4.60 \times 10^4$ viable counts of the strain Ravenel were infected intravenously.

* Figures represent average of 5 animals $\pm$ standard deviation.

These results are in accord with those reported by Kun and Miller (12) in the intoxication of rabbits with the Salmonella endotoxin.

*Effect of the extraction of heat-killed virulent tubercle bacilli with petrol-ether:* It may be seen in table 4 that the virulent tubercle bacilli lose their effect on the liver succinic dehydrogenase activity when they are repeatedly extracted with large amounts of petrol-ether. Then, the extracted bacilli of the virulent human tubercle bacilli H37Rv were coated with cord factor[3] according to the method described by Bloch (3), and the effect on the liver succinic dehydrogenase activity was again tested by injecting 1.0 mg. of the cord-factor–coated bacilli. The succinic dehydrogenase activity of the liver decreased significantly this time. Although bacterial constituents other than cord factor must also be examined for their effect on the liver succinic dehydrogenase activity, this fact seems to verify definitively that the cord factor is the bacterial constituent active for the decrease of succinic dehydrogenase activity of the liver of tuberculous mice.

*Comparison of the alteration of the enzymic activity of the liver of mice between mice inoculated with Ehrlich's ascites tumor cells and of animals in-*

[3] Obtained through the courtesy of Dr. H. Bloch, Department of Microbiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania.

TABLE 3

EFFECT OF HEAT-KILLED BACTERIA OF VARIOUS MICROBIAL STRAINS ON SUCCINIC DEHYDROGENASE ACTIVITY OF MOUSE LIVER

| Bacterial Strains Injected | Dry Weight of Inoculum | $\gamma$ of Formazan Reduced per Mg. N per Min. (Mean ± Standard Deviation) |
|---|---|---|
| | *mg.* | |
| *M. tuberculosis* (bovine Ravenel) | 0.3 | 1.16 ± 0.78 Significant ($\alpha$ = 0.01) |
| *Salmonella typhi* (H901 W) | 0.3 | 1.25 ± 0.81 Significant ($\alpha$ = 0.01) |
| *Micrococcus pyogenes* var. *aureus* (209 P) | 0.3 | 3.82 ± 0.78 Not significant |
| Control | Saline, 0.3 ml. | 3.44 ± 1.36 |
| *Brucella mellitensis* (No. 4) | 1.0 | 5.10 ± 1.65 Not significant |
| *Bacillus anthracis* (1 Byo) | 0.1 | 4.64 ± 0.84 Not significant |
| *Shigella dysenteriae* (Shiga) | 1.0 | 5.78 ± 1.12 Not significant |
| Control | Saline, 0.2 ml. | 4.72 ± 0.81 |

Five animals in each group. The determination of succinic dehydrogenase activity of the liver homogenate was done 24 hours after the intravenous injection of heat-killed bacteria.

TABLE 4

EFFECT OF EXTRACTION WITH PETROL-ETHER AND COATING WITH CORD FACTOR OF TUBERCLE BACILLI ON THE ACTIVITY TO DECREASE SUCCINIC DEHYDROGENASE ACTIVITY OF MOUSE LIVER

| Mice Received Intravenously 1.0 Mg. Dry Weight of: | Number of Mice | $\gamma$ of Formazan Reduced per Mg. per Min. (Mean ± Standard Deviation) |
|---|---|---|
| H37Rv, Nonextracted | 5 | 1.96 ± 0.36 Significant ($\alpha$ = 0.01) |
| Control, saline 0.2 ml. | 5 | 2.78 ± 0.29 |
| H37Rv, Extracted with petrol-ether | 5 | 2.94 ± 0.32 Not significant |
| Control, saline 0.2 ml. | 5 | 2.84 ± 0.27 |
| H37Rv, extracted with petrol-ether and coated with cord factor | 5 | 1.64 ± 0.37 Significant ($\alpha$ = 0.01) |
| Control, saline 0.2 ml. | 5 | 2.84 ± 0.27 |

Bacilli were extracted with 10 volumes of petrol-ether for five successive times. Cord factor was dissolved in ether and coated onto the bacilli according to Bloch's method. The determination of the succinic dehydrogenase of mouse liver was done 24 hours after the injection.

*fected with tubercle bacilli:* In table 5 are shown the dehydrogenase activities of the liver of mice which were inoculated with a heavy dose of Ehrlich's ascites tumor nine days before the determination of enzymic activity. Succinic, lactic, malic, and alanine dehydrogenase activities are, notwithstanding their moribund state, normal in comparison with those of control animals.

The succinic dehydrogenase activity and the catalase activity of cord-factor–treated, tuberculous, and tumor-bearing mice are compared in table 6. Succinic dehydrogenase decreased after injection of cord factor but not after inoculation of ascites tumor. Catalase, on the contrary, decreased in the liver of tumor-bearing mice but not after injection of cord factor.

Interestingly enough, both enzymic activities

TABLE 5

DEHYDROGENASE ACTIVITY IN LIVER OF MICE BEARING EHRLICH'S ASCITES TUMOR

| Substrate | Number of Mice | $\gamma$ of Formazan Reduced per Mg. N per Min. (Mean ± Standard Deviation) | |
|---|---|---|---|
| | | Normal Control | Tumor-bearing |
| Succinate | 6 | 1.92 ± 0.39 | 2.25 ± 1.32 |
| Lactate | 6 | 0.26 ± 0.14 | 0.35 ± 0.29 |
| Malate | 6 | 0.34 ± 0.19 | 0.41 ± 0.16 |
| L-Alanine | 6 | 0.47 ± 0.14 | 0.54 ± 0.11 |

The difference between control and tumor-bearing mice was examined and found to be non-significant. The determination of enzymic activity was done nine days after the intraperitoneal inoculation of ascitic cells.

TABLE 6

COMPARISON OF BIOCHEMICAL DISTURBANCE IN MOUSE LIVER BETWEEN EXPERIMENTAL TUBERCULOSIS AND EHRLICH'S ASCITES TUMOR

| Treatment (Number of Animals Used) | Succinic Dehydrogenase (Mean γ of Formazan Reduced per Mg. N per Min. ± Standard Deviation) | Catalase (Mean Catalase Activity ± Standard Deviation) |
|---|---|---|
| Cord factor* (10) | 2.99 ± 0.57 Significant ($\alpha$ = 0.01) | 58.2 ± 2.1 Not significant |
| Olive oil control (10) | 5.08 ± 0.94 | 55.2 ± 6.9 |
| Infected with H37Rv† (5) | 2.86 ± 0.78 Significant ($\alpha$ = 0.01) | 48.9 ± 23.7 Significant ($\alpha$ = 0.01) |
| Saline control (5) | 4.34 ± 0.65 | 97.3 ± 31.8 |
| Infected and tuberculin-shocked‡ (5) | 3.36 ± 0.55 Significant ($\alpha$ = 0.01) | 38.6 ± 7.7 Significant ($\alpha$ = 0.01) |
| Saline control (5) | 4.34 ± 0.65 | 97.3 ± 31.8 |
| Inoculated with Ehrlich's ascites tumor§ (5) | 2.25 ± 1.32 Not significant | 19.7 ± 3.9 Significant ($\alpha$ = 0.01) |
| Normal control (5) | 1.92 ± 0.39 | 55.2 ± 20.7 |

* 0.1 mg. of cord factor was dissolved in 0.1 ml. of olive oil and injected into mice intraperitoneally.
† 1.0 mg. wet weight of H37Rv was injected intravenously 55 days before.
‡ 0.2 ml. of undiluted Old Tuberculin was injected intravenously 7 hours before.
§ Nine days after the inoculation of ascites cells.

were significantly decreased in the late stage of tuberculosis. Mice infected with virulent tubercle bacilli received, fifty-five days later, 0.2 ml. of a nondiluted Old Tuberculin (OT) (prepared from a six-week culture of H37Rv in Sauton medium) by the intravenous route. Seven hours after the tuberculin injection, the succinic dehydrogenase and the catalase activities were determined. The former enzymic activity was not affected specifically by the injection of OT, but the difference of the catalase activity between the infected mice and the infected and tuberculin-treated mice was significant. These facts seem to mean that the decrease of the catalase activity in tuberculous infection may be caused in relation to an allergic tissue reaction. This result is in accord with those previously reported by Yamamura and associates (8) on the liver catalase activity in rabbits.

## DISCUSSION

Bacterial infection may alter the metabolic pattern of the infected tissues in any one of the following three ways:

1. Under a direct or indirect action of toxic bacterial constituents on an enzyme or enzyme system of host cells, as in the case of diphtheria (13, 14), gas gangrene (15), meningococcal and Salmonella infection (12, 16, 17), Lansing and Theiler FA viral infection (18), and tuberculosis (1, 2).

2. Through a metabolic competition between bacteria and host cells for some essential metabolites or through the production of some anti-metabolites by bacteria which interfere with the essential metabolic activity of host cells during the survival and multiplication of the infecting agents in host tissues. The type classified in this category of bacterial infection is: infection with *Pasteurella tularensis* (19), *Fusarium lycopersici* (20), and *Pseudomonas tabaci* (20, 21). The recent study of the loss and recovery of virulence by the simultaneous injection with purine derivatives of purine-requiring mutants of *Salmonella typhi* (22, 23) and *Klebsiella pneumoniae* (24) for mice are clear examples of competition between bacteria and host for growth factors. But the mechanism of competition between host and bacteria is not satisfactorily understood in most of the above mentioned cases.

3. Through an indirect interference with host metabolism by some bacterial constituents or, perhaps, tissue degradation products, based upon an altered host tissue reaction, such as phenomena termed "allergy" or "immunity." This type of biochemical dis-

turbance produced by bacterial infection has been infrequently investigated because of the extreme complexity of the phenomenon and the lack of the knowledge of chemistry of either the relating tissue factors or bacterial constituents. Chaudhuri and Martin (25) and Martin and associates (26) are considering that the decrease of the succinic dehydrogenase activity in the kidneys of tuberculous guinea pigs occurs in some relation to the tuberculin allergy. The present writers, dealing with a similar phenomenon in tuberculous mice, could not reach the same conclusion. As will be discussed later, the decrease of the catalase activity by tuberculous infection could be accounted for by the tuberculin allergy. The fact supporting this view was previously reported with tuberculous rabbits by Yamamura and associates (8).

In the present paper the biochemical disturbance of the mouse liver produced by the infection with tubercle bacilli was investigated. The decrease of the succinic dehydrogenase activity by the injection of cord factor which was reported previously (1) was experimentally reproduced by either the injection of heat-killed tubercle bacilli or by infection with living virulent bacteria.

The reducing activity of virulent strains of tubercle bacilli on the succinic dehydrogenase is lost by the extraction of the bacteria with petrol-ether and is reproduced by coating the strains with cord factor. Thus, it appears likely that the cord factor is at least one of the active principles which plays a role in the decrease of the succinic dehydrogenase activity of liver in murine tuberculosis. The possibility remains, however, that some other bacterial constituents would have the same biochemical activity. This is now being investigated.

In the three living infections with Ravenel, H37Rv, and BCG, the degree of the decrease of succinic dehydrogenase activity is closely parallel with the severity of the infection, suggesting that the decrease of succinic dehydrogenase activity of liver is an important biochemical alteration of host tissues, determining the severity and fatality of the infection of mice with virulent tubercle bacilli.

Considering the fact that the succinic dehydrogenase activity is decreased by the injection of heat-killed cells of either virulent tubercle bacilli or *Salmonella typhi*, it seems that many bacterial constituents and many different mechanisms would exist to disturb the activity of the succinic dehydrogenase of infected host tissues. Kun and Miller (12) described the inhibiting effect of the endotoxins of Salmonella and Meningococcus. The comparative investigation of the mechanisms of inhibition reaction between tuberculosis and other infections seems to yield a better understanding in the biochemistry of bacterial infections.

It seems also very interesting that Brucella undergoes an intracellular multiplication in the same manner as *Mycobacterium tuberculosis*, but the injection of its heat-killed bacilli are ineffective in causing inhibition of the succinic dehydrogenase of the mouse liver. Infections with Brucella and *Mycobacterium tuberculosis* have many features in common, as is well established, but they seem to differ essentially in the metabolic interaction with host cells.

The study of the biochemical disturbance in mice bearing Ehrlich's ascites tumor presents some new interesting problems. The catalase activity of liver was markedly decreased, as is well established; but the succinic, malic, lactic, and alanine dehydrogenase activities were not affected despite the dying state of tumor-bearing animals. This fact seems to provide evidence that the decrease of the succinic dehydrogenase activity of the mouse liver by the injection of cord factor or by infection with virulent tubercle bacilli is not due to a decline of the metabolic activity of the host cells in general, but to a specific biochemical disturbance based upon a specific biochemical interaction between bacteria and host.

It seems of interest that the injection of cord factor caused a marked decrease of succinic dehydrogenase without affecting the enzymic activity of catalase, while in murine tuberculosis both enzymic activities were similarly decreased. A finer analysis of the phenomenon is now proceeding, but there is little doubt that the succinic dehydrogenase activity is decreased under the action of cord factor, which is the primary toxic bacterial constituent; and the catalase activity is affected by the allergic tissue reaction caused by tuberculoprotein. The latter assumption is confirmed also by a previous report by Yamamura and associates (8) that the catalase activity of rabbit liver is markedly decreased by the injection of tuberculin into mesenteric veins of hypersensitized animals. The claim by Martin and

co-workers (26) that the succinic dehydrogenase activity was decreased in the kidney by the tuberculin shock is not confirmed, at least in murine tuberculosis. It is possible that this lack of agreement of the results is due to the difference of the reaction of animals to tuberculous infection, since Martin and co-workers were working with guinea pigs to analyze the phenomenon.

In murine tuberculosis, both succinic dehydrogenase and catalase activities of the liver decrease. It seems legitimate from the experimental results described in the present paper that the decrease of the succinic dehydrogenase activity depends on the toxic effect of cord factor and that the decrease in catalase activity is due to some allergic tissue reactions.


## Summary

The biochemical disturbance produced in the infection of mice with virulent tubercle bacilli was studied. The succinic dehydrogenase activity of the liver of mice was decreased by the intravenous injection of virulent bovine (Ravenel) and human (H37Rv) tubercle bacilli but not by the attenuated bovine (BCG), avirulent human (H37Ra) tubercle bacilli, and saprophytic mycobacteria (*M. phlei*, *M. smegmatis*, *M. 607*, and *M. butyricum*).

The decrease of succinic dehydrogenase of liver closely paralleled the development of a fatal virulent infection of mice with the highly virulent bovine tubercle bacilli (Ravenel). A hardly recognizable decrease of the same enzymic activity was noted with BCG which soon returned to normal activity. The effect of the virulent tubercle bacilli, which caused the decrease of succinic dehydrogenase of mouse liver, disappeared by the extraction of bacilli with petrol-ether and again appeared by coating bacilli with cord factor.

The biochemical disturbance of the liver of mice between Ehrlich's ascites tumor and tuberculous infection was compared. The succinic dehydrogenase was decreased in infection with virulent tubercle bacilli but not in mice with ascites tumor. The catalase activity was decreased in both cases. The decreased catalase activity of tuberculous mice was further decreased by the injection of Old Tuberculin, while the succinic dehydrogenase activity was not subjected to the additional effect of the injection of tuberculin.

In murine tuberculosis, the succinic dehydrogenase in liver is decreased by the toxic effect of cord factor, while the catalase is affected by the tuberculous allergy in a later stage of infection.



## Sumario

### *Análisis Bioquímico de la Virulencia de los Bacilos Tuberculosos*

Este estudio comprende el trastorno bioquímico producido al infectar ratones con bacilos tuberculosos virulentos. La actividad de la dehidrogenasa succínica del hígado de los ratones fué reducida por la infección intravenosa con bacilos tuberculosos bovinos (Ravenel) y humanos (H37Rv), pero no por los bacilos tuberculosos bovinos atenuados (BCG), humanos avirulentos (H37Ra) y micobacterias saprofitas (*M. phlei*, *M. smegmatis*, *M 607* y *M. butyricum*).

La disminución de la dehidrogenasa succínica del hígado reflejó de cerca la evolución de una infección virulenta letal de los ratones con bacilos tuberculosos bovinos sumamente virulentos (Ravenel). Con el BCG se notó una disminución apenas reconocible de la misma actividad enzímica, que pronto retornó a lo normal. El efecto de los bacilos tuberculosos virulentos, que ocasionó la disminución de la dehidrogenasa succínica del hígado murino, desapareció con la extracción de los bacilos con petróleo-éter para aparecer de nuevo al recubrir los bacilos con el factor cordón.

Se comparó el trastorno bioquímico del hígado murino en el tumor seítico de Ehrlich y la infección tuberculosa. La dehidrogenasa succínica disminuyó en la infección por bacilos tuberculosos virulentos, pero no en los ratones con el tumor ascítico. La actividad de la catalasa disminuyó en ambos casos. La disminuída actividad de la catalasa en los ratones tuberculosos fué reducida aun más por la inyección de tuberculina antigua, mientras que la actividad de la dehidrogenasa succínica no se mostró sometida al efecto sobrepuesto de la inyección de tuberculina.

En la tuberculosis murina, la dehidrogenasa succínica del hígado es disminuída por el efecto tóxico del factor cordón, en tanto que la catalasa se ve afectada por la alergia tuberculosa en un período más tardío de la infección.



### *Acknowledgment*

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# STUDIES ON THE BIOLOGIC PROPERTIES OF MYCOBACTERIOPHAGE[1, 2]

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## INTRODUCTION

Four mycobacteriophages active against tubercle bacilli of both human and bovine origin have been reported by Froman and associates (1). Recently, several mycobacteriophages have been isolated by the present writers (2), and some of these isolated phages were found to lyse a large range of tubercle bacilli. In the present paper, the biologic properties of these phages will be reported.

## MATERIALS AND METHODS

*Mycobacteria used:* Fifty-five strains of saprophytic mycobacteria, 14 strains of *M. avium*, one strain of *M. muris*, 13 strains of *M. tuberculosis* var. *bovis*, and 26 strains of *M. tuberculosis* var. *hominis*, from stock cultures of the Bacteriological Department, School of Medicine, Kyushu University, were used for susceptibility tests. Among 14 strains received labeled as *M. avium*, colonies of 6 strains were of the R type, while 8 strains showed S-type colonies.

Besides the stock cultures mentioned above, 18 strains of drug-resistant human tubercle bacilli isolated from tuberculous patients in a sanatorium and 8 attenuated human tubercle bacilli strains of Imamura (3) together with their original virulent strains were examined to clarify the relationship between drug resistance, virulence, and phage susceptibility.

*Bacteriophages:* A soil enrichment technique (1, 2) was used for the isolation of phages. Soil samples were collected from a flower bed, a rice field, a chicken house, and several vegetable gardens, all fertilized with human or chicken feces. Mycobacteriophages were successfully isolated from all samples of fertilized soil. On the contrary, no phage was isolated from two unfertilized garden soil samples.

The isolated phage strains were designated as B-1, B-2, B-3, A-2, A-3, A-4, A-5, A-6, and A-7. The sources and the host strains of these phages are shown in table 1. Host cells from the solid media were cultivated in 4 per cent glycerol broth containing 0.004 M calcium chloride for eight to fourteen hours with aeration. Phage suspension was added to this culture and the culture was again incubated for ten to twenty-four hours. The lysate was centrifuged for thirty minutes at 3,000 r.p.m., and the supernatant was filtered through a bacterial filter. The final filtrate contained approximately $10^8$ to $10^9$ phage particles per ml. This stock phage was stored at 4°C. and was used for further experiments.

Each phage was revealed by the electron microscope to be tadpole shaped. The detailed electron microscope study will be reported elsewhere (4).

*Bacteriophage susceptibility test:* A modification of the agar layer method of Froman and associates (1) (which is called "spot test" in this paper) was used for demonstrating phage susceptibility. An 0.3-ml. dispersed suspension of each test organism (grown in Dubos broth base medium for saprophytic bacilli or Dubos Tween®-albumin medium for pathogenic tubercle bacilli) was mixed with 2.5 ml. of 0.7 per cent semi-solid agar medium containing 4 per cent glycerol and 0.004 M calcium chloride. The mixtures containing the saprophytic bacilli were layered on 4 per cent glycerol agar medium, and the mixtures containing the pathogenic tubercle bacilli were layered on Ogawa egg medium (5). The plates were incubated zero to two hours for saprophytes, zero to three days for *M. avium*, and three to five days for *M. tuberculosis* var. *bovis* and *hominis*. At this time a loopful of each stock phage suspension containing approximately $10^8$ phage particles per ml. was spotted on each of the plates. All of the plates were again incubated and observed until growth became visible.

*Neutralization test:* Anti-phage serum was prepared in rabbits by injecting phage filtrates at three-day intervals for two months. The anti-phage titer of serum prepared in this experiment was rather low (*see* table 5). The tests were carried out by the method of Burnet (6). Dilutions of anti-phage serum were made in 4 per cent glycerol broth. To each of these tubes, diluted phage equivalent to half the serum volume was added. After these mixtures were incubated at 37°C. for two hours, the number of plaque-forming particles was counted.

*Animal inoculation of Mycobacterium avium:* All cultures were grown on Ogawa egg medium. Ten milligrams of bacilli suspended in saline were injected subcutaneously into four-week-old white leghorn chickens, and intravenously into rabbits weighing 1.5 to 2.0 kg. Three of 27 chickens and 2 of 12 rabbits died during the course of the experiment. The remaining animals were sacrificed about four months after inoculation, and the extent of involvement of various organs was evaluated macroscopically. After weighing, smears

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[2] Presented at the 33rd Annual Meeting of the Japanese Association for Tuberculosis, Osaka, May 24–26, 1958.

TABLE 1

MYCOBACTERIOPHAGES, SOURCES, HOST STRAINS, AND ACID-FAST BACILLI USED FOR ENRICHMENT

| Group | Acid-fast Bacilli Used for Enrichment | Soil Sample | Name of Phage | Host Strain |
|---|---|---|---|---|
| B | *M. sp.* (Kido)<br>*M. avium* (Jucho) | Flower bed | B-1 | *M. avium* (Jucho) |
| | *M. avium* (Takeo)<br>*M. avium* (A62) | Vegetable garden (2)* | B-2 | *M. avium* (Jucho) |
| | *M. avium* (A71) | Vegetable garden (3) | B-3 | *M. avium* (Jucho) |
| A | *M. smegmatis* | Vegetable garden (1) | A-2 | *M. smegmatis* |
| | *M. phlei* | Vegetable garden (2) | A-3 | *M. sp.* ATCC 607 |
| | *M. sp.* ATCC 607 | Vegetable garden (3) | A-4 | *M. phlei* |
| | *M. sp.* (Kaeru-Jikei) | Vegetable garden (4) | A-5 | *M. sp.* (Kaeru-Jikei) |
| | | Rice field | A-6 | *M. sp.* ATCC 607 |
| | | Flower bed | A-7 | *M. phlei* |
| | | Garden (1) | — | — |
| | | Garden (2) | — | — |

* Numbers in parentheses = number of soil samples.

were made from each organ and examined for acid-fast bacilli. Cultures for the bacilli were made from the spleen and liver. The organs were then fixed with formalin for further histologic examination.

## RESULTS

*Plaque morphology:* The size and shape of plaque of each phage is shown in figure 1 and table 2. Phages B-1, B-2, and B-3 presented similar plaque morphology. The plaque size of A-6 was largest. The plaque appearance of A-4 was slightly hazy, but the plaques of the other phages appeared clear.

*Susceptibility of acid-fast bacilli to isolated phages:* Bacteriophage susceptibility tests revealed that B-1, B-2, B-3, A-2, and A-3 were active against virulent human tubercle bacilli. The lytic spectrum is given in table 3. B-1 had the broadest host range (figure 2).

Fourteen strains were received labeled avian tubercle bacilli. Eight of these strains (Jueki, Flamingo, A71, 4110, 4121(A), 4121(B), 3717(A), and 3717(B)) had the colonial and cultural characteristics typical of avian tubercle bacilli except that most of the colonies of the Jueki and Flamingo strains were of the umbilicated, flat, smooth type. The colonies of 5 of the strains (Jucho, Takeo, Denken, Dencho, and A62(C)) were of the atypical spreading R or RS types. They grew rapidly within one week. The A62 strain, which was originally isolated from spontaneous avian tuberculosis and which was highly virulent when tested formerly, showed the R-type colony; its growth required at least three to four weeks. Typical strains were hardly susceptible to all of the isolated phages, while most of the atypical strains were lysed by some of the phages.

The virulence tests made on chickens and rabbits revealed that typical strains caused progressive tuberculosis in chickens, and Yersin or progressive tuberculosis in rabbits, but atypical strains were avirulent or weakly virulent to chickens and rabbits. Although the Jueki and Flamingo strains of typical avian tubercle bacilli were found to be positive to the spot test for determining the susceptibility to B-1 or other phages, constant positive results were obtained only when more than $10^8$ phage particles per ml. were used for the spot test. Furthermore, plaques could not be detected by the agar layer method. This will indicate that growth inhibition might have occurred at the spotted place instead of lysis of bacteria. The fact that the phage suspension purified by ultra-centrifugation also inhibited the growth of the bacilli by the spot test suggests that this growth inhibition was due to the adsorption of phage particles to the bacilli.

As the Jueki strain was found to contain a considerable number of R-type colonies besides the S-type colonies, it was used for isolating R- and S-type colonies. The S-type dissociant was less susceptible than the R-type dissociant. The S-type dissociant of the Jucho strain was also tested for susceptibility to phage lysis and

FIG. 1. Plaque morphology of the isolated phages.

*A*: B-1 on host strain *M. avium* (Jucho).
*B*: B-2 on host strain *M. avium* (Jucho).
*C*: A-2 on host strain *M. smegmatis*.
*D*: A-3 on host strain *M. sp.* ATCC 607.
*E*: A-4 on host strain *M. phlei*.
*F*: A-5 on host strain *M. sp.* (Kaeru-Jikei).
*G*: A-6 on host strain *M. sp.* ATCC 607.
*H*: A-7 on host strain *M. phlei*.

TABLE 2

PLAQUE MORPHOLOGY OF THE ISOLATED PHAGES AFTER TWENTY-FOUR HOURS' INCUBATION

| Phage | Size in Mm. | Appearance | Border |
|---|---|---|---|
| B-1 | 1.5–3.0 | clear | regular |
| B-2 | 1.5–3.0 | clear | regular |
| B-3 | 1.5–3.0 | clear | regular |
| A-2 | 0.5–1.5 | clear | regular |
| A-3 | 1.5–3.0 | clear | regular |
| A-4 | 1.0–2.0 | slightly hazy | regular |
| A-5 | 0.5–1.0 | clear | regular |
| A-6 | 2.0–6.0 | clear | irregular |
| A-7 | 0.3–1.0 | clear | regular |

found to be less susceptible than its original R-type strain (figure 3).

In order to isolate phage strains lytic against typical avian tubercle bacilli, 3 typical avian tubercle bacilli and 2 saprophytic acid-fast bacilli with smooth colonies were added to three soil samples for enrichment of phage. Three mycobacteriophages (designated C-1, C-2, and C-3) were isolated, but none of these were lytic to the typical avian tubercle bacilli, although C-3 inhibited the growth of 4 of 8 typical strains.

*Classification of isolated phages:* As *Mycobacterium sp.* ATCC 607 was found to be susceptible to all of the phage strains except A-5, a cross-susceptibility test was carried out using resistant strains of this bacterium to each phage. Some of the phage-resistant strains showed colonial morphology different from that of the original strain. The result of this test showed that B-1, B-2, A-2, and A-3 were closely related, but that A-4, A-6, and A-7 were different from this group (table 4).

The neutralization test with antiserum against B-1 is summarized in table 5. Phages B-1, B-2, and B-3 were serologically hardly distinguishable; A-2 was closely related to B-1; and A-3 was also somewhat related to this group. The remaining phage strains were serologically different from B-1. Phages D28, D29, D32, and D34 of Froman and co-workers (1) were also tested and found to be serologically different from B-1.

*Susceptibility of drug-resistant acid-fast bacilli:* No difference in phage susceptibility was found between strains of *Mycobacterium sp.* ATCC 607 susceptible and resistant to 200 $\gamma$ of isoniazid and susceptible and resistant to 1,000 $\gamma$ of streptomycin. Eighteen drug-resistant strains of human tubercle bacilli isolated from tuberculous patients were also examined for phage susceptibility. These strains were resistant to either isoniazid, PAS, or streptomycin, or were resistant to two of these drugs in varying degrees. The phage-susceptibility patterns, however, were almost uniform among these resistant strains and susceptible stock cultures.

*Virulence and phage susceptibility:* Eight strains of attenuated human tubercle bacilli of Imamura obtained by successive cultures on Calmette's bile medium for 250 generations (3) were all found to be less susceptible to phages than were their original virulent strains (table 6). The attenuated strains had more or less flat, spreading, irregular, and wrinkled colonies on egg medium, although colonies of the original strains were typically rough, irregular, raised, and granular. A BCG strain with colonial appearance similar to the above-mentioned attenuated strains was also less susceptible than the virulent bovine tubercle bacilli. On the contrary, phage-susceptibility patterns of H37Rv and H37Ra were almost identical. The difference

TABLE 3

SUSCEPTIBILITY OF STOCK CULTURE ACID-FAST BACILLI TO ISOLATED PHAGES

| Phage | Number of Strains Positive to Spot Susceptibility Test | | | | | |
|---|---|---|---|---|---|---|
| | Tubercle Bacilli | | | | | Saprophytic Acid-fast Bacilli |
| | Human | Bovine | Murine | Avian | | |
| | | | | Typical* | Atypical | |
| | Number of strains tested | | | | | |
| | 26 | 13 | 1 | 8 | 6 | 55 |
| B-1 | 25 | 12 | 1 | 2 | 6 | 34 |
| B-2 | 25 | 11 | 1 | 1 | 6 | 22 |
| B-3 | 25 | 11 | 1 | 1 | 6 | 17 |
| A-2 | 25 | 9 | 1 | 1 | 6 | 19 |
| A-3 | 24 | 0 | 0 | 0 | 4 | 14 |
| A-4 | 0 | 0 | 0 | 0 | 4 | 15 |
| A-5 | 0 | 0 | 0 | 0 | 0 | 8 |
| A-6 | 0 | 0 | 0 | 0 | 3 | 5 |
| A-7 | 0 | 0 | 0 | 0 | 1 | 11 |

* Susceptibility of typical avian tubercle bacilli does not necessarily mean lysis by the phages.

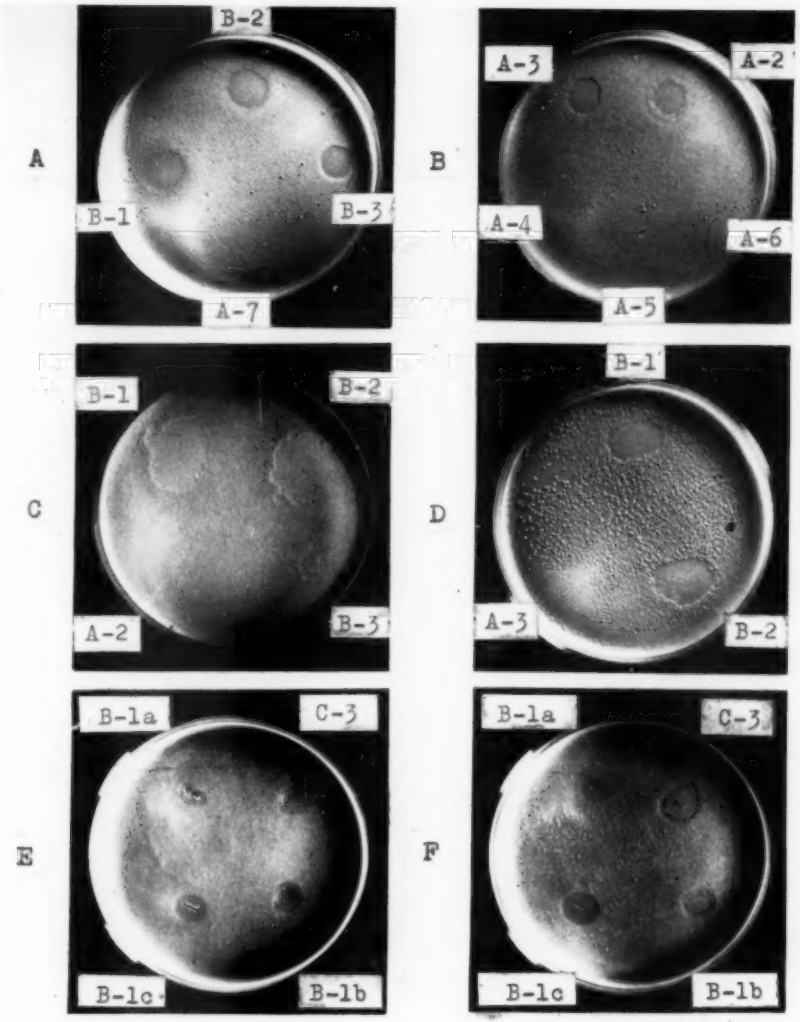


FIG. 2. Patterns of phage susceptibility as shown by the "spot test."

*A* and *B: M. tuberculosis* var. *hominis* (H37Rv).

*C: M. tuberculosis* var. *bovis* (Miwa).

*D: M. muris.*

Phage suspensions containing approximately $10^8$ phage particles per ml. were used for *A*—*D*.

*E: M. avium* (Jueki).

*F: M. avium* (Flamingo).

*C-3:* $5 \times 10^9$ C-3 phage particles per ml.

*B-1a:* $3 \times 10^7$ B-1 phage particles per ml.

*B-1b:* $3 \times 10^9$ B-1 phage particles per ml.

*B-1c:* $1 \times 10^{10}$ B-1 phage particles per ml.

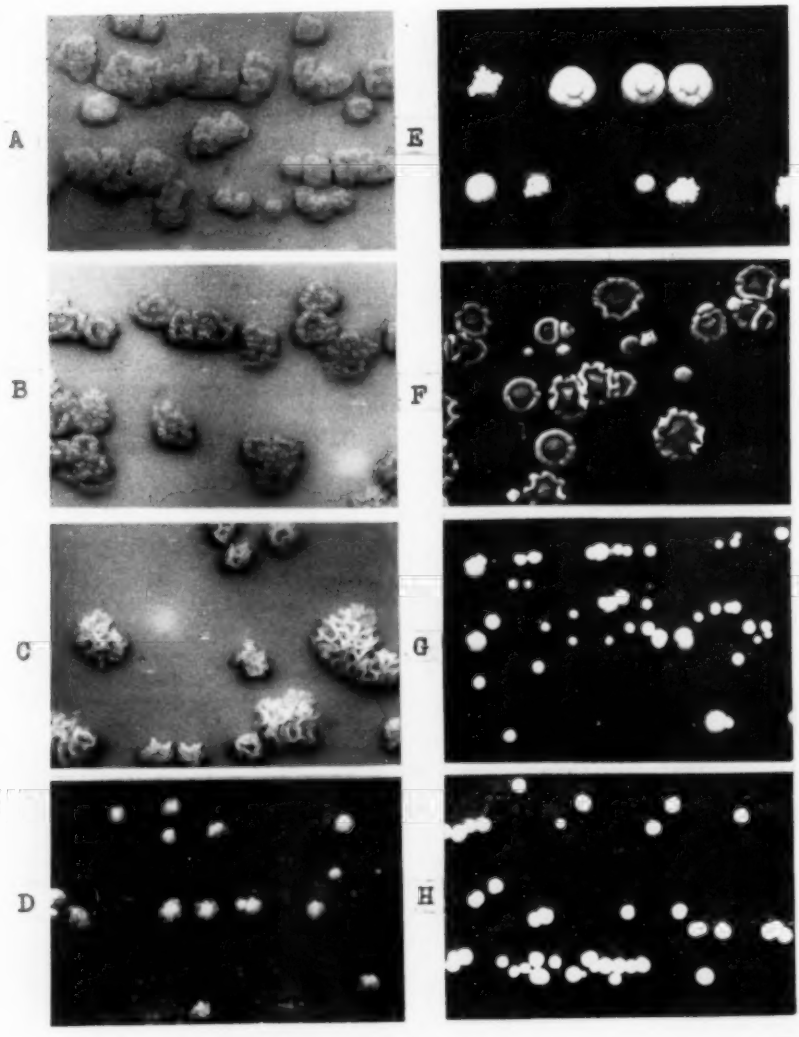


FIG. 3. Colonies of several strains received labeled as avian tubercle bacilli on Ogawa egg medium.
*A:* Jucho strain. *B:* S-type dissociant of Jucho strain.
*C:* Takeo strain. *D:* A62 strain. *E:* Jueki strain.
*F:* Flamingo strain. *G:* 4110(A) strain. *H:* A71 strain.
*A—C:* 5-day culture. *D—H:* 4-week culture.

in colonial morphology between the H37Rv and H37Ra strains was not so marked as that between the virulent and attenuated strains of Imamura (figure 4).

*Lysogenization:* An attempt was made to detect lysogenic strains among saprophytic acid-fast bacilli. Twenty strains of saprophytic acid-fast bacilli were cultivated in 4 per cent glycerol broth and the culture filtrates were spotted on plates previously inoculated with various strains

TABLE 4

CROSS-SUSCEPTIBILITY PATTERN OF PHAGE-RESISTANT MYCOBACTERIUM SP. ATCC 607

| Strain | Colonial Morphology | Phage | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | B-1 | B-2 | B-3 | A-2 | A-3 | A-4 | A-6 | A-7 |
| Original | R-type, yellowish brown | ††† | ††† | ††† | ††† | ††† | ††† | ††† | ††† |
| 607 (B-1)* | SR-type, lightly yellow | — | — | — | — | — | ††† | ††† | ††† |
| 607 (B-2) | SR-type, lightly yellow | — | — | — | — | — | ††† | ††† | ††† |
| 607 (B-3) | S-type, yellowish white | — | — | — | — | — | ††† | ††† | ††† |
| 607 (A-2) | R-type, yellowish brown | — | — | — | — | — | ††† | ††† | ††† |
| 607 (A-3) | SR-type, lightly yellow | — | — | — | — | — | ††† | ††† | ††† |
| 607 (A-4) | R-type, yellowish brown | ††† | ††† | ††† | ††† | ††† | — | ††† | ††† |
| 607 (A-6) | R-type, lightly yellowish brown | ††† | ††† | ††† | ††† | ††† | ††† | — | — |
| 607 (A-7) | R-type, yellowish brown | ††† | ††† | ††† | ††† | †† | ††† | ††† | — |

* 607(*#*) = *Mycobacterium sp.* ATCC 607 resistant to phage *#*.
††† = Complete lysis.
†† = Lysis with resistant colonies.
— = No lysis and no growth inhibition.

of acid-fast bacilli. However, among the cultures so far examined, lysogenesis could not be demonstrated.

Culture filtrates of phage-resistant strains of *Mycobacterium sp.* 607 grown in Dubos broth base medium in five generations were examined for their lytic activity using *Mycobacterium sp.* 607 as the indicator strain. Strains resistant to A-2 and A-4 were found to have lytic activity. Since Tween 80 is known to prevent the adsorption of phage to bacteria (7–9), single colonies of both strains were further cultivated in Dubos broth base medium for ten generations, and plaque counts were made by the agar layer method for each generation. The result, as may be seen in table 7, shows that the number of plaques per milliliter of the culture filtrate for each generation was approximately constant.

To eliminate contaminating phage particles, the free phage particles in the culture of *Mycobacterium sp.* 607 resistant to A-2 were almost completely neutralized with anti-phage serum. After neutralization, the culture was streaked on 4 per cent glycerol agar to obtain isolated colonies. A single colony was cultivated in Dubos broth base medium, and plaque count was again made on the culture filtrate. The amount of free phage particles in the culture filtrate was constant even after neutralization with antiserum.

## DISCUSSION

Soil samples fertilized with human or chicken feces appear to be good sources of mycobacteriophage, as shown by the fact that 12 strains of mycobacteriophage have been isolated by 12 soil enrichments in this experiment. If different acid-fast bacilli had been employed for enrichment, different phages (B-1 and A-7, B-2 and A-3, B-3 and A-4) could have been isolated from the same soil samples (*see* table 1). On the other hand, serologically indistinguishable phage strains (B-1, B-2, and B-3) were isolated from different soil samples when the same acid-fast bacilli were used for enrichment. These data may be useful in considering the origin of the isolated phages.

Will and co-workers (10) reported that true avian tubercle bacilli were not susceptible to

TABLE 5

NEUTRALIZATION TEST OF ISOLATED PHAGES WITH ANTI-B-1 SERUM

| Phage | Plaque Counts after Neutralization | | | | | | Plaque Counts of Controls |
|---|---|---|---|---|---|---|---|
| | Dilution of Anti-B-1 Serum | | | | | | |
| | 12.5× | 25× | 50× | 100× | 200× | 400× | |
| B-1 | | | 0.5 | 3 | 14 | 29.5* | 114 |
| B-2 | | | 0.5 | 5.5 | 18 | 56* | 253 |
| B-3 | | | 0 | 2 | | 23.5* | 92.5 |
| A-2 | | | 2 | 8 | 30* | | 115 |
| A-3 | | | 26.5* | 72.5 | 107 | 116.5 | 166.5 |
| A-4 | 104 | | | | | | 96 |
| A-5 | 119 | | | | | | 94 |
| A-6 | | 79 | | | | | 88.5 |
| A-7 | 63 | | | | | | 60 |

* 75 per cent reduction of the plaque count as compared with that given by controls.

TABLE 6

SUSCEPTIBILITY OF VIRULENT AND ATTENUATED STRAINS OF HUMAN TUBERCLE BACILLI OF IMAMURA TO B-1, A-2, AND A-3

| Phage | Virulent Strains | | | | | | | | Attenuated Strains | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. 1* | No. 2 | No. 3 | No. 4 | No. 5 | No. 6 | No. 7 | No. 8 | No. 1 | No. 2 | No. 3 | No. 4 | No. 5 | No. 6 | No. 7 | No. 8 |
| B-1 | ††† | ††† | ††† | ††† | ††† | ††† | ††† | ††† | †† | † | † | †† | † | †† | ††† | †† |
| A-2 | ††† | †† | ††† | ††† | ††† | †† | †† | †† | † | † | † | — | † | — | — | † |
| A-3 | † | † | † | †† | † | † | † | † | — | † | † | — | — | — | — | — |

* No. 1 to No. 8 strains correspond respectively to the Kimoji, Sonoda, Nooka, Aoki, Yamaguchi, Toda, Tatsumi, and Kanazawa strains.

††† = Complete lysis.

†† = Lysis with resistant colonies.

† = Several plaques or growth inhibition at the spotted place.

— = No lysis and no growth inhibition.

lysis by the four phages isolated by them, and that phage typing would be valuable in the screening of possible avian tubercle bacilli. In the present experiment, also, all of the typical avian tubercle bacilli could not be lysed by any of the isolated phages, but the growth of a few of these strains was inhibited by some of the isolated phages. This growth inhibition may probably be due to the abortive infection. Atypical avian tubercle bacilli[3] with R-type colonies, in contrast to typical avian tubercle bacilli, were highly susceptible to lysis by isolated phages. In general, acid-fast bacilli of S-type colonies seem to be less susceptible to phages than those of the R-type colonies.

Phage-susceptibility patterns of drug-susceptible and -resistant strains were almost uniform among the acid-fast bacilli so far examined. However, further studies would be necessary to confirm the relationship between the phage susceptibility and drug resistance of acid-fast bacilli.

Froman and associates (11) have examined in detail the biologic characteristics and the bacteriophage susceptibility of various strains of BCG, H37Rv, H37Ra, and bovine tubercle bacilli. They reported that the H37Rv and H37Ra strains were all highly and uniformly susceptible to lysis by phage, but that none of the BCG strains tested showed as high and as consistent susceptibility. In the present experiment, attenuated strains of human tubercle bacilli of Imamura were found to be less susceptible to the isolated phages than their original virulent strains. This difference in phage susceptibility, however, might not be related to virulence itself because the H37Rv and H37Ra strains showed an almost identical susceptibility pattern. This difference might be explained by the changes in colonial morphology, since the colonial appearance of attenuated strains was remarkably different from that of the original virulent

[3] The writers believe that these bacilli may possibly not be avian tubercle bacilli. However, in this experiment they were tentatively designated as atypical avian tubercle bacilli because they had been received labeled as avian tubercle bacilli. Further experiments on the drug-susceptibility test (PAS, isoniazid, and neotetrazolium) and cross-tuberculin reactions using purified protein $\pi$ (12) prepared from the culture filtrate of each strain have disclosed that the A62 strain is a weakly-virulent true avian tubercle bacillus with rough colonial growth; but the remaining 5 strains, which grew rapidly, did not resemble typical avian tubercle bacilli in most characteristics.

TABLE 7

NUMBER OF PLAQUES PER ML. OF FILTRATES OF THREE-DAY CULTURE OF PHAGE-RESISTANT MYCOBACTERIUM SP. ATCC 607 IN DUBOS BROTH BASE MEDIUM

| Generations in Dubos Broth Base Medium | *Mycobacterium Sp.* 607 Resistant to A-2 | *Mycobacterium Sp.* 607 Resistant to A-4 |
|---|---|---|
| 1 | $3.0 \times 10^3$ | $1.6 \times 10^3$ |
| 2 | $1.4 \times 10^3$ | $2.0 \times 10^3$ |
| 3 | $1.6 \times 10^3$ | $1.9 \times 10^3$ |
| 4 | $1.2 \times 10^3$ | $1.5 \times 10^3$ |
| 5 | $4.6 \times 10^3$ | $6.5 \times 10^3$ |
| 6 | $3.6 \times 10^{3*}$ | $4.7 \times 10^3$ |
| 7 | $1.9 \times 10^3$ | $5.4 \times 10^3$ |
| 8 | $3.6 \times 10^3$ | $4.6 \times 10^3$ |
| 9 | $2.5 \times 10^3$ | $5.7 \times 10^3$ |
| 10 | $3.8 \times 10^3$ | $6.2 \times 10^3$ |

* Neutralization with anti-phage serum.

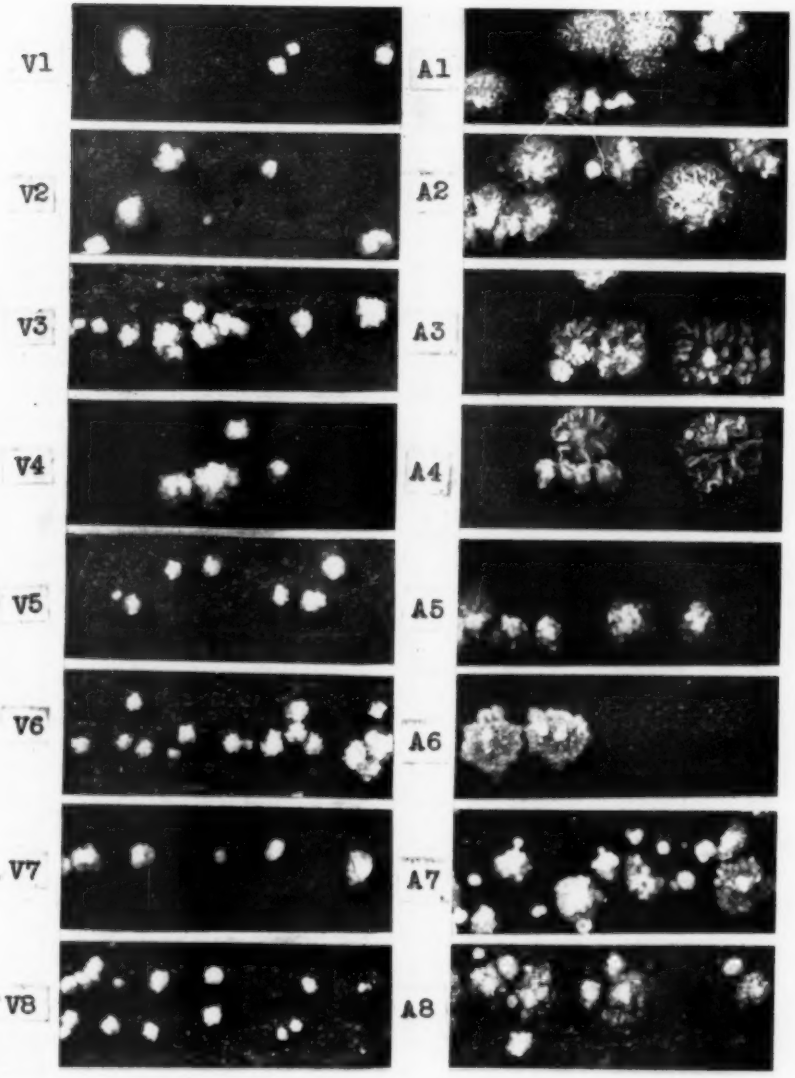


FIG. 4. Colonies of virulent and attenuated strains of Imamura on Ogawa egg medium.
*V1—V8:* Virulent original strains No. 1 to No. 8.
*A1—A8:* Attentuated strains No. 1 to No. 8.

strains. The fact that the S-type dissociants of avian tubercle bacilli were less susceptible to phages than the R-type dissociants or their original strains of the R type may support this explanation. Although colonies of H37Rv and H37Ra tubercle bacilli were reported to be sharply distinguishable (11), the difference in the colonial appearance of these strains was not so marked in this experiment.

Naturally lysogenic strains could not be detected among the acid-fast bacilli so far examined. *Mycobacterium sp.* ATCC 607 resistant

to phages A-2 or A-4 released an approximately constant number of free phage particles in the culture filtrate of Dubos broth base medium for each generation even after neutralization with anti-phage serum. This fact may suggest that these strains had become lysogenic, since Tween 80 contained in Dubos broth base medium is known to inhibit the adsorption of phage to bacteria (7–9). This evidence for the probable existence of lysogenesis in acid-fast bacilli will promise a prospective field for further investigation with mycobacteriophage.


## Summary

The biologic properties of 9 recently isolated mycobacteriophages are reported.

A neutralization test with anti-B-1 serum revealed that the phages B-1, B-2, and B-3 were serologically hardly distinguishable; A-2 was closely related to B-1; A-3 was somewhat related to this group; and the rest of the isolated phages were entirely different. The data of cross-susceptibility tests, using phage-resistant *Mycobacterium sp.* ATCC 607, agreed with this result.

Phages B-1, B-2, B-3, and A-2 were lytic against human and bovine tubercle bacilli, and A-3 was lytic against human tubercle bacilli. Atypical avian tubercle bacilli, which had R-type colonial morphology and were avirulent or weakly virulent to chickens and rabbits, were easily lysed by many of the isolated phages. Typical strains of avian tubercle bacilli, however, were not susceptible to lysis, although some of them were susceptible to growth inhibition by some of the phage strains.

The phage-susceptibility patterns of drug-susceptible and drug-resistant acid-fast bacilli were not different among the strains so far examined. Attenuated strains of human tubercle bacilli of Imamura were less susceptible than the original virulent strains. However, it was suggested that this difference in susceptibility might be based on the change in colonial morphology because the susceptibility patterns of H37Rv and H37Ra were almost identical.

Naturally lysogenic strains could not be detected among the 20 saprophytic acid-fast bacilli examined. On the other hand, *Mycobacterium sp.* ATCC 607, resistant to A-2 and A-4, was found to release an approximately constant number of free phage particles for each generation of the culture in Dubos broth base medium, even after neutralization with anti-phage serum.

## Sumario

### *Estudios de las Propiedades Biológicas del Micobacteriófago*

Discútense las propiedades biológicas de 9 micobacteriófagos recién aislados.

Una prueba de neutralización con el antisuero B-1 reveló que el B-1, el B-2 y el B-3 eran apenas distinguibles serológicamente; que el A-2 estaba íntimamente relacionado con el B-1; que el A-3 estaba algo relacionado con este grupo; y que los demás bacteriófagos aislados eran absolutamente distintos. Los datos arrojados por las pruebas de susceptibilidad cruzada, usando *Mycobacterium sp.* ATTC 607, fagorresistente, convinieron con dicho resultado.

El B-1, el B-2, el B-3 y el A-2 se mostraron líticos contra los bacilos tuberculosos bovinos y el A-3 lítico contra los los bacilos tuberculosos humanos. Los bacilos tuberculosos aviarios atípicos, cuyas colonias tenían una morfología de forma B, y que eran avirulentos o débilmente virulentos para los pollos y los conejos, fueron lisados fácilmente por muchos de los bacteriófagos aislados. Sin embargo, las típicas cepas de bacilos tuberculosos aviarios no fueron susceptibles a la lisis, aunque algunas de ellas se mostraron susceptibles a la inhibición de su desarrollo por algunas de las cepas de fagos.

El patrón de la susceptibilidad a los bacteriófagos de los bacilos fármacosusceptibles y fármacorresistentes no fué distinto en las cepas estudiadas hasta ahora. Las cepas atenuadas de los bacilos tuberculosos humanos de Imamura fueron menos susceptibles que las primitivas cepas virulentas. No obstante, se apunta que esta diferencia en la susceptibilidad podría deberse a la alteración de la morfología colonial visto que fueron casi idénticos los patrones de susceptibilidad del H37Rv y del H37Ra.

No pudieron descubrirse cepas naturalmente lisógenas entre los bacilos ácidorresistentes saprofíticos estudiados. En cambio, se observó que el *Mycobacterium sp.* ATCC 607, resistente al A-2 y al A-4, liberaba una cantidad aproximadamente constante de partículas bacteriofágicas libres en cada generación del cultivo en el medio a base de caldo de Dubos, aun después de la neutralización con suero antibacteriofágico.

## Resume

### *Etudes sur les propriétés biologiques des mycobactériophages*

Les propriétés biologiques de 9 mycobactériophages recemment isolés sont rapportées.

Un test de neutralisation avec un sérum anti-B-1 a révélé que B-1, B-2 et B-3 étaient à peu

près indistinguibles sérologiquement; A-2 était très proche de B-1; B-3 était légèrement apparenté à ce groupe; les autres phages isolés étaient entièrement différents. Les données relatives aux tests de sensibilité croisée utilisant le *Mycobactérium sp.* ATCC 607, confirmaient ce résultat.

B-1, B-2, B-3 et A-2 étaient lytiques vis-à-vis des bacilles tuberculeux humains et bovins et A-3 était lytique vis-à-vis des bacilles tuberculeux humains. Les bacilles tuberculeux aviaires atypiques, dont la morphologie était celle des colonies type R, et qui étaient avirulents ou faiblement virulents pour les poulets et les lapins, étaient facilement lysés par un grand nombre des phages isolés. Les souches types de bacilles tuberculeux aviaires étaient cependant réfractaires à la lyse, bien que quelques uns d'entre eux puissent subir une inhibition de la croissance par certaines des souches de phages.

Les modalités de la sensibilité au phage des bacilles acido-résistants sensibles, ou résistants aux drogues, ne présentaient aucune différence quant aux souches examinées jusqu'à présent. Les souches atténuées de bacilles tuberculeux humains de Imamura étaient moins sensibles que les souches virulentes originales. Toutefois, on avait l'impression que cette différence dans la sensibilité pouvait être basée sur une modification de la morphologie des colonies, car les modalités de la sensibilité de H37Rv et de H37Ra étaient presque identiques.

Parmi les 20 bacilles acido-résistants saprophytes examinés on n'a pas décelé de souches naturellement lysogéniques. D'autre part on a observé que le *Mycobactérium sp.* ATCC 607, résistant à A-2 et à A-4, libérait un nombre approximativement constant de particules phage libres pour chaque génération de la culture en milieu Dubos à base de bouillon, même après neutralisation avec un sérum anti-phage.

*Acknowledgment*

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# REINFECTION IN GUINEA PIGS WITH TUBERCULOSIS ARRESTED BY CHEMOTHERAPY[1]

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## INTRODUCTION

It has been established (1–3) that tuberculous animals become resistant to reinfection provided they are not treated. If the tuberculous animal is treated and reinfected, however, it still shows some degree of resistance, judged from the survival of such a group when compared with untreated, infected controls (4–8). Furthermore, it has been shown that a high reinfecting dose will produce a generalized tuberculosis in guinea pigs previously infected and treated with isoniazid (10). The present investigation was carried out in order to clarify the following three points: (*1*) if there is generalization of the reinfecting tubercle bacilli when small inocula are used; (*2*) if repeated successive small inocula can produce tuberculosis in this type of experimental animal; (*3*) if reinfection will activate the tubercle bacilli which were introduced into the animal as first infection and which have been arrested in the regional lymph nodes by the effect of chemotherapy.

## MATERIAL AND METHODS

The H37Rv strain of *Mycobacterium tuberculosis* and its viomycin-resistant variant (Vio-R) were used throughout the experiments. The viomycin-resistant strain was obtained by successive transfers in Löwenstein-Jensen medium containing increasing amounts of viomycin, and has been maintained in this laboratory for over a year without any apparent change in resistance. At the time it was used, the viomycin-resistant strain was resistant to 100$\gamma$ of viomycin per ml. of medium, and susceptible to both isoniazid and dihydrostreptomycin.

Tuberculin-negative guinea pigs weighing 300 to 350 gm. were distributed according to the following outline:

*Experiment No. 1.* Reinfection in animals with progressive tuberculosis:

(*a*) Four animals were infected with 0.2 mg. of H37Rv (wet weight) and sacrificed after sixty days (infection controls).

(*b*) Eight animals infected as in (*a*) were reinfected with the viomycin-resistant strain (0.2 mg.) sixty days after the first infection, and sacrificed in pairs ten, fifteen, twenty, and thirty days after reinfection.

*Experiment No. 2.* Reinfection in animals with tuberculosis treated with chemotherapy:

Twenty-eight animals were infected with 0.2 mg. of H37Rv (wet weight). Treatment was started on the following day and continued for sixty days. The regimen consisted of the oral administration of 10 mg. of isoniazid daily and the parenteral administration of 12 mg. of dihydrostreptomycin three times a week. At the end of this period the animals were separated into the following groups:

(*a*) Four animals were sacrificed at the end of treatment (treatment controls).

(*b*) Eight animals were reinfected with 0.02 mg. of the viomycin-resistant strain (wet weight) at the end of treatment and sacrificed in pairs at ten, fifteen, twenty, and thirty days after reinfection.

(*c*) Eight animals were reinfected twice with 0.02 mg. of the viomycin-resistant strain (wet weight) at intervals of five days, and sacrificed following the schedule of group (*b*) after the second reinfection.

(*d*) Eight animals were reinfected three consecutive times with 0.02 mg. of the viomycin-resistant strain at intervals of five days between each reinfection, and sacrificed in the same way as group (*c*) after the third reinfection.

*Experiment No. 3.* Controls of infection with the viomycin-resistant strain:

(*a*) Eight animals were inoculated with 0.02 mg. of the viomycin-resistant strain (wet weight) and sacrificed in pairs at ten, fifteen, twenty, and thirty days after infection.

(*b*) Eight animals were inoculated twice with 0.02 mg. of the viomycin-resistant strain (wet weight) each time, with an interval of five days between infections, and sacrificed as was group (*a*).

(*c*) Eight animals were inoculated three consecutive times with 0.02 mg. of the viomycin-resistant strain (wet weight), with an interval of five days between each infection, and sacrificed following the schedule of group (*a*).

All inoculations were made subcutaneously: first infections in the inner aspect of the left thigh, and reinfections in the inner aspect of the right thigh. Bacterial counts were made in Hopkins tubes.

*Bacteriologic and pathologic studies:* All guinea pigs were examined at autopsy, and smears and cultures in Löwenstein-Jensen medium were pre-

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TABLE 1

AUTOPSY FINDINGS IN GUINEA PIGS WITH TUBERCULOSIS ARRESTED BY CHEMOTHERAPY AND REINFECTED WITH THE VIOMYCIN-RESISTANT STRAIN

| Groups | Findings | Lymph Nodes | | | Spleen | Liver | Lungs |
|---|---|---|---|---|---|---|---|
| | | R.F.I.* | R.R.† | Rp.‡ | | | |
| With tuberculosis arrested by treatment with isoniazid and dihydrostreptomycin during 60 days | Macroscopic lesions | ± | − | − | − | − | − |
| | Microscopic lesions | ± | − | − | − | − | − |
| | Culture | ± | − | − | − | − | − |
| | Isolated strain | H37Rv | − | − | − | − | − |
| With tuberculosis arrested by treatment with isoniazid and dihydrostreptomycin during 60 days, and reinfected once with 0.02 mg. of the viomycin-resistant strain | Macroscopic lesions | ± | + | + | − | − | − |
| | Microscopic lesions | ± | + | + | − | − | − |
| | Culture | + | + | + | + | − | − |
| | Isolated strain | H37Rv | Vio-R§ | Vio-R | Vio-R | − | − |
| With tuberculosis arrested by treatment with isoniazid and dihydrostreptomycin during 60 days, and reinfected twice with 0.02 mg. of the viomycin-resistant strain | Macroscopic lesions | ± | + | + | ± | − | − |
| | Microscopic lesions | + | + | + | +′ | +′ | +′ |
| | Culture | + | + | + | + | + | +′ |
| | Isolated strain | H37Rv | Vio-R | Vio-R | Vio-R | Vio-R | Vio-R |
| With tuberculosis arrested by treatment with isoniazid and dihydrostreptomycin during 60 days, and reinfected three times with 0.02 mg. of the viomycin-resistant strain | Macroscopic lesions | ± | + | + | +′ | ± | ± |
| | Microscopic lesions | + | + | + | +′ | +′ | +′ |
| | Culture | + | + | + | + | +′ | +′ |
| | Isolated strain | H37Rv | Vio-R | Vio-R | Vio-R | Vio-R | Vio-R |
| With progressive tuberculosis, and reinfected once with 0.2 mg. of the viomycin-resistant strain | Macroscopic lesions | + | + | + | + | + | + |
| | Microscopic lesions | + | + | + | + | + | + |
| | Culture | + | + | + | + | + | + |
| | Isolated strain | H37Rv | Vio-R | H37Rv | H37Rv | H37Rv | H37Rv |

* R.F.I = Regional, first infection.
† R.R. = Regional, reinfection.
‡ Rp. = Retroperitoneal.
§ Vio-R = Viomycin-resistant strain.
(+) = Presence of lesions; positive culture.
(−) = No lesions; negative culture.
(′) = One animal was negative in this organ.
(±) = Some animals were negative in this organ.

pared using one-half of the following organs: lymph nodes, spleen, liver, and lungs. The other half of these organs was used for the preparation of microscopic slides with hematoxylin and eosin.

The cultures were examined every week and, when positive, the susceptibility to viomycin was determined in all except those obtained from the control groups. A tuberculin test was carried out

in all animals thirty and sixty days after starting treatment with a 1:10 dilution of crude tuberculin.

## Results

*Experiment 1* (infected untreated animals challenged sixty days after inoculation with 0.2 mg. of the viomycin-resistant strain): The infected controls inoculated with the H37Rv strain showed generalized tuberculosis. In the animals with progressive tuberculosis which were reinfected, it was not possible to recover the reinfection strain from any of the organs studied; it was found only at the site of reinfection.

*Experiment 2* (infected treated animals challenged sixty days after one, two, and three inoculations with 0.02 mg. of the viomycin-resistant strain at five-day intervals): Animals infected and treated for sixty days, when sacrificed at the end of that period, failed to show both gross and microscopic changes except in a few cases in which there were minimal, fibrous lesions of the regressive type involving the lymph nodes draining the site of inoculation.

Animals reinfected once showed viomycin-resistant bacilli in both the lymph nodes draining the site of inoculation and the spleen at ten, fifteen, twenty, and thirty days after reinfection; the cultures of the liver and lung were consistently negative at all times. The histopathologic examination revealed tuberculous lesions in the draining lymph nodes and no changes in the spleen, in spite of the fact that bacilli were recovered from the latter organ; there were no lesions in the lung and in the liver. When the animals were reinfected two or three times, it was possible to demonstrate the presence of bacilli as well as of tissue changes in all of the organs examined. The cultures revealed a greater number of colonies the longer the survival period of the animals sacrificed.

Most of the animals gave a positive tuberculin reaction of variable intensity before reinfection (+ to ++++); however, there were 5 animals with negative reactions.

*Experiment 3* (animals infected one, two, and three times with 0.02 mg. of the viomycin-resistant strain at five-day intervals; reinfection controls): The viomycin-resistant strain was isolated from all organs studied in these animals ten days after inoculation; tuberculous lesions were readily apparent both grossly and microscopically, and increased in intensity with the prolongation of the time after inoculation.

The results obtained in the reinfected groups appear in table 1, and the pathologic findings are presented in table 2.

TABLE 2

Histopathologic Study of Animals Reinfected with the Viomycin-resistant Strain

| Animals | Microscopic Lesions | | | |
|---|---|---|---|---|
| | Regional Lymph Nodes | Spleen | Liver | Lungs |
| With progressive tuberculosis and reinfected | Extensive granulomas with caseous necrosis | Granulomas with extensive necrosis | Granulomas with scarce necrosis | Granulomas with necrosis |
| With tuberculosis arrested by chemotherapy and reinfected once | Extensive granulomas with caseous necrosis | No lesions | No lesions | No lesions |
| With tuberculosis arrested by chemotherapy and reinfected twice | Extensive granulomas with caseous necrosis | Granulomas with scarce to extensive necrosis | Granulomas (′) | Granulomas with scarce necrosis (′) |
| With tuberculosis arrested by chemotherapy and reinfected three times | Extensive granulomas with caseous necrosis | Granulomas with extensive necrosis | Granulomas with scarce necrosis (′) | Granulomas with necrosis |
| With tuberculosis arrested by chemotherapy. Non-reinfected controls | Granulomas with necrosis | No lesions | No lesions | No lesions |

(′) = One animal showed no lesions in these organs.

## Discussion

Previous experiments have demonstrated that in guinea pigs inoculated with tubercle bacilli and treated with isoniazid and dihydrostreptomycin, the infection will remain localized in the lymph nodes draining the site of inoculation, and that no dissemination will occur for as long as one hundred and twenty days after discontinuation of treatment (9). If these animals were reinfected with a strain resistant to some agent different from those used in the treatment, it would be relatively simple to identify the reinfecting organism. A previous communication from this laboratory has given support to such a theoretic deduction (10) and the present work has taken advantage of it.

The results of these experiments reveal a difference in the outcome of reinfection in treated, as compared with untreated, animals. In guinea pigs with treated tuberculosis, the reinfecting (viomycin-resistant) strain was recovered from several organs; in the untreated animals, the reinfecting microorganism was blocked and even eliminated from the site of inoculation (3). Therefore, dissemination of reinfecting bacilli in treated animals is obvious, while in untreated animals it is absent. This difference is all the more striking when it is considered that most animals in both groups showed a positive tuberculin reaction. It is worthy of consideration that in the treated group there were 5 animals with negative tuberculin reactions at the time of reinfection and that the findings in these animals were, nevertheless, the same as in those with positive tuberculin reactions. This fact may support the idea that, although hypersensitivity is associated with some infections, it plays no role in the defense of the organism against such infections. This fact is difficult to interpret because the tuberculin reaction in guinea pigs is variable and not very strong. Other writers have shown that there is no direct relationship between the degree of hypersensitivity and the degree of acquired resistance (11–13), and that the hypersensitivity is not responsible for the lack of dissemination of tubercle bacilli in animals with acquired resistance (14). Furthermore, the fact that some infected and treated animals show positive tuberculin reactions of different intensities, while others give negative reactions, may throw some light on the role played by hypersensitivity in the formation of tubercles and in the presence of caseous necrosis; it seems feasible that these lesions may appear without the participation of hypersensitivity. However, these results are not conclusive, and more work will be necessary before any definite conclusion can be reached in this problem.

Animals infected and treated under the conditions of the present experiment show a certain degree of resistance evidenced by the lesser degree of dissemination as well as by the number and extension of the lesions of reinfected animals when compared with the infected controls. Guinea pigs treated and reinfected only once will show bacilli in the spleen ten days after reinfection, but no lesions will develop even at thirty days after reinfection. On the other hand, controls infected with a similar dose develop lesions in all organs ten days after inoculation, and tubercle bacilli can be recovered from them. It appears, therefore, that the accelerated tubercle formation does not appear to be the only mechanism of resistance.

It does not seem logical that the animals with progressive tuberculosis should be more capable of inhibiting dissemination of a large number of bacilli while the animals treated are unable to inhibit dissemination of a small number of microorganisms. It might be suggested that the degree of resistance of a tuberculous animal is very high and, therefore, could accomplish the destruction *in situ* (15) of bacilli. By contrast, a lesser degree of resistance could eliminate only a few of the reinoculated bacilli and would permit the dissemination of the remainder. It is also possible that in both cases there is a rapid dissemination to many organs, but bacilli are immediately destroyed in tissues with extensive lesions while the absence or small size of the lesions in treated animals permits multiplication of microorganisms. If this were so, it would be a case of interference of reproduction of reinfected bacilli by tissues extensively damaged.

The degree of resistance of animals with treated tuberculosis was overwhelmed by two or three consecutive reinfections; in these cases the disease was similar to the one present in infected controls.

Reinfection did not modify the status of the first infection; in no case was there dissemination of bacilli corresponding to the first inoculation.

## Summary

An experimental study has been made of the effect of reinfection on guinea pigs with tubercu-

losis arrested by chemotherapy. By means of small inocula (0.02 mg.), it has been shown that treated, tuberculous animals acquire a certain degree of resistance to reinfection manifested by the absence of lesions in the internal organs. However, dissemination is not inhibited since tubercle bacilli can be recovered from some of the same organs. Successive reinfections will overcome this resistance and produce a generalized disease similar to that in the control animals. The state of the first infection is not modified by reinfection.


## Sumario

*Reinfección en los Cobayos con Tuberculosis Estacionada por la Quimioterapia*

Se ha verificado un estudio experimental del efecto de la reinfección en los cobayos que tienen tuberculosis estacionada por la quimioterapia. Por medio de pequeños inóculos (0.02 mg.) se ha demostrado que los animales tuberculosos tratados adquieren cierta resistencia a la reinfección, traducida por la falta de lesiones en las vísceras. Sin embargo, no se inhibe la diseminación dado que pueden obtenerse bacilos tuberculosos de algunos de los mismos órganos. Las reinfecciones sucesivas vencerán esta resistencia y producirán una enfermedad generalizada semejante a la de los animales testigos. El estado de la primera infección no es modificado por la reinfección.

## Resume

*Réinfection des cobayes après tuberculose jugulée par la chimiothérapie*

Il a été procédé à une étude expérimentale sur les effets de la réinfection du cobaye dont la tuberculose a été arrêtée par la chimiothérapie. Au moyen de petits inocula (0,02 mg), il a été démontré que les animaux tuberculeux traités acquièrent un certain degré de résistance à la réinfection, résistance manifestée par l'absence de lésions dans les organes internes. Néanmoins la dissémination n'est pas inhibée car les bacilles tuberculeux peuvent être recouvrés à partir de certains de ces mêmes organes. Les réinfections successives supprimeront cette résistance et produiront une maladie généralisée semblable à celle des animaux témoins. L'état de l'infection primitive n'est pas modifié par la réinfection.


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# AMITHIOZONE: MECHANISM OF ACTION AND RESISTANCE DEVELOPMENT BY MYCOBACTERIA[1, 2]

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## INTRODUCTION

The problem of microbial resistance to drugs has been of interest to investigators from the time of Ehrlich. However, more serious consideration has been given to this subject in recent times with a view to learning not only the biologic aspect of how the resistant organisms can dispose of the drug, but also to gain an insight into the mode of action in order to develop newer related chemotherapeutic agents with greater effect.

There are but a few instances in which the mechanism of action of drugs on the bacterial cell appears to be well understood. The interaction between sulphanilamide and *p*-aminobenzoic acid is one among these. Practically nothing is known about the specific action of amithiozone on certain types of tubercle bacilli. The biologic mechanisms relating to drug resistance, although complicated, are rendered easier to follow if the cells also show some biochemical alteration besides resistance. Thus, the demonstration by Middlebrook (1) of the loss of catalase by isoniazid-resistant mutants was a great lead to further investigations. This also led the present writers to investigate peroxidase, an enzyme closely related to catalase. It was shown that there is a striking relationship between the occurrence of peroxidase in tubercle bacilli and their susceptibility to isoniazid (2). Another type of biochemical alteration with amithiozone resistance made the present study feasible.

During investigations on the metabolism of mycobacteria, it was observed that an amithiozone-resistant mutant of *M. tuberculosis* H37Rv (H37Rv-AmR) produced, like the naturally resistant saprophytes, a yellow pigment which diffused into the medium, while the parent susceptible strain did not. This departure of the mutant from its parent to produce such a coloration, besides resistance, incited great interest. In this connection, two possibilities as to the nature of the pigment itself were considered; if amithiozone interfered with the respiratory mechanism of the susceptible bacteria, it was not unlikely that the selected resistant mutant produced greater amounts of a flavine nucleotide to counteract the metabolic insufficiency in the oxidation-reduction processes; second, the pigment may be quite unrelated to the action of the drug itself and may simply be a by-product in a blocked reaction, with the function carried through an alternate metabolic pathway. Consideration of these two possibilities, in addition to those critically evaluated by Davis and McDermott (3), led the writers to a "metabolic area" where amithiozone exerts its activity.

## MATERIALS AND METHODS

The following strains of microorganisms have been used in this study:

*M. tuberculosis:*

- H37Rv[3]
- H37Rv-INHR (resistant to 100 $\gamma$ of isoniazid per ml.)
- H37Rv-StrR (resistant to 100 $\gamma$ of streptomycin per ml.)
- H37Rv-PASR (resistant to 100 $\gamma$ of PAS per ml.)
- H37Rv-AmR (resistant to 100 $\gamma$ of amithiozone per ml.)
- H37Rv (NCTC 7416)
- H37Rv NCTC-AmR (resistant to 100 $\gamma$ of amithiozone per ml.)
- Ravenel[3]
- Ravenel-INHR (resistant to 100 $\gamma$ of isoniazid per ml.)
- Ravenel-StrR (resistant to 100 $\gamma$ of streptomycin per ml.)
- Ravenel-AmR (resistant to 100 $\gamma$ of amithiozone per ml.)
- Ravenel-PASR (resistant to 100 $\gamma$ PAS per ml.)
- H37Ra[3]
- BCG (Jensen)
- BCG-AmR (resistant to 100 $\gamma$ of amithiozone per ml.)
- E 10883-human[4]
- E 10883-AmR (resistant to 100 $\gamma$ of amithiozone per ml.)

[1] From the Research Laboratories of J. R. Geigy S. A., Basle, Switzerland.

[2] Presented in part at the Seventeenth Annual Meeting of the Swiss Microbiological Society, Rheinfelden, June 28, 1958.

[3] Kindly supplied by Dr. W. Steenken, Jr., Trudeau Laboratory, Saranac Lake, New York.

[4] Kindly supplied by Dr. H. C. Engbaek, Statens Serum Institute, Copenhagen, Denmark.

A 5-human (laboratory strain)
A 5-AmR (resistant to 100 γ of amithiozone per ml.)
415-AmR[5] (resistant to 100 γ of amithiozone per ml.)
511-AmR[5] (resistant to 100 γ of amithiozone per ml.)
B-9-AmR[6] (resistant to 100 γ of amithiozone per ml.)
B101-AmR[6] (resistant to 100 γ of amithiozone per ml.)
B180-AmR[6] (resistant to 100 γ of amithiozone per ml.)
Sheard[3]
GV 7081[7] (*avium*)
GV 5418[7] (*avium*)
GV 8645[7] (*avium*)
*M. smegmatis* (NCTC 8152) (saprophyte)
*M. butyricum* (NCTC 333) (saprophyte)
*M. lacticola* $O_{11}$[8] (saprophyte)

The mutant strains were produced in the laboratory by passage *in vitro* on Löwenstein-Jensen medium containing different amounts of amithiozone or other drugs. In general, strains resistant to amithiozone were also resistant to several other thiosemicarbazone derivatives investigated. In addition to those listed, streptomycin- or isoniazid-resistant mutants were also made resistant to amithiozone, and their properties were studied.

The organisms were cultivated on Löwenstein-Jensen medium or Dubos Tween®-albumin medium and the experiments were carried out with Youmans medium (4), omitting glycerol or asparagin when studying the influence of carbon or nitrogen source, respectively. The rate of growth with *meso*-inositol was considerably slowed down with all strains; therefore, in later experiments, *meso*-inositol was added at 1 per cent final concentration without omitting glycerol. Purines and pyrimidines were incorporated without omitting any constituent of the medium. After separate sterilization, 6-mercaptopurine, dissolved in alkali, and 2,6-diaminopurine in acid were added to Youmans medium. Pigment production was noted usually at the end of thirty days' incubation at 37°C.

*Riboflavin* analysis of the pigmented culture filtrates was carried out by the standard fluorimetric procedure (5) using a Lumetron Fluorescence Meter, Model 402-EF. Briefly, the method involved hydrolysis of the filtrates with dilute hydrochloric acid at 100°C., neutralization with trisodium phosphate, removal of precipitated proteins by centrifugation, destruction of interfering fluorescent substances by oxidation with 5 per cent potassium permanganate, and removal of the excess of the latter with dilute hydrogen peroxide. The solution made up to a definite volume was analyzed for its content of the vitamin. The difference in optical density of the yellow fluorescence excited with a radiation between 400 and 420 mμ in the presence and absence of added dithionite was considered to be due to riboflavin. But the extract showed a blue fluorescence instead of yellow, and this was not significantly affected by dithionite.

*Flavin-adenine-dinucleotide* (FAD) in the heated or partially hydrolyzed culture filtrates was determined by the Warburg technique, using kidney D-amino acid oxidase[9] and DL-alanine[10] as the substrate. Standard solutions of the crystalline cofactor, FAD,[9] stimulated the uptake of oxygen for the oxidation of alanine by the enzyme.

*Deaminases of adenine and adenosine* in mycobacteria were detected as follows: Bacteria were harvested by centrifugation of approximately 10-day-old cultures on Dubos medium, washed and re-suspended in Sørensen phosphate buffer of pH 6.8 containing 0.025 per cent Tween 80. One milliliter of such a suspension containing about 600,000 bacteria (MacFarland No. 2) was mixed with 1 ml.

TABLE 1

PIGMENT PRODUCTION BY MYCOBACTERIA ON YOUMANS AND DUBOS MEDIA

| Strain | Youmans Medium | Dubos Tween-Albumin Medium with: At 1 Per Cent | | | |
|---|---|---|---|---|---|
| | | None | Glucose | Glycerol | *Meso*-Inositol |
| *M. tuberculosis* H37Rv | − | − | − | − | − |
| *M. tuberculosis* H37Rv-AmR | ++ | − | ++ | ++ | +++ |
| *M. tuberculosis* Ravenel | − | − | − | − | − |
| *M. tuberculosis* Ravenel-AmR | − | − | − | − | − |
| *M. tuberculosis* Sheard | − | − | − | − | − |
| *M. smegmatis* (saprophyte) | +++ | − | ++ | +++ | ++++ |
| *M. butyricum* (saprophyte) | +++ | − | ++ | +++ | ++++ |
| *M. lacticola* (saprophyte) | ++ | − | ++ | ++ | ++++ |

− = No pigmentation; + to ++++ = increasing amounts of pigment formation.

[5] Kindly supplied by Dr. R. Bönicke, Tuberkulose-Forschungsinstitut, Borstel bei Bad Oldesloe, Germany.

[6] Kindly supplied by Dr. K. Bartmann, Landes Tuberkulosekrankenhaus Heckeshorn, Berlin-Wannsee, Germany.

[7] Obtained from the Institut Galli-Valério, Lausanne, Switzerland.

[8] Kindly supplied by Dr. J. V. Bhat, Indian Institute of Science, Bangalore, India.

[9] Nutritional Biochemicals Inc.

[10] Roche.

TABLE 2

EFFECT OF CARBON SOURCES ON PIGMENT PRODUCTION BY MYCOBACTERIA ON YOUMANS MEDIUM

| Strain | Pigment Production with: At 2 Per Cent Concentration | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | D (+)Mannose | D (−)Fructose | D (+)Glucose | D(−) Ribose | D (+)Galactose | L (−)Xylose | L (+)Rhamnose | Sucrose | Maltose | Lactose | Raffinose | L (−)Sorbase | Glycerol | *Meso*-Inositol |
| *M. tuberculosis* H37Rv | − | − | − | (+) | − | (−) | − | − | − | − | − | − | − | (−) |
| *M. tuberculosis* H37Rv-AmR | − | − | + | (++) | ± | (+) | + | − | ± | − | − | − | ++ | (+++) |
| *M. tuberculosis* Ravenel | − | − | − | (+) | − | − | − | − | − | − | − | − | − | (−) |
| *M. tuberculosis* Ravenel-AmR | − | − | − | (+) | − | − | − | − | − | − | − | − | − | (−) |
| *M. tuberculosis* Sheard | − | − | − | (+) | (−) | (−) | (−) | (−) | (−) | (−) | (−) | − | − | (−) |
| *M. smegmatis* (saprophyte) | + | + | + | ++ | + | ++ | + | − | − | − | − | ++ | +++ | ++++ |
| *M. butyricum* (saprophyte) | + | + | ++ | ++ | + | ++ | + | − | ± | − | − | − | +++ | ++++ |
| *M. lacticola* (saprophyte) | + | + | ± | +++ | ± | ++ | ± | − | ± | − | − | − | +++ | ++++ |

− = No pigment; + to ++++ = increasing amounts of pigmentation; ( ) = no growth.

of either adenine (1 mg.) or adenosine (2 mg.), and incubated at 37°C. for three hours. Then 1 ml. of Nessler's reagent was added, and the yellowish-red color due to liberation of ammonia was noted.

## RESULTS

The results of an observation of preliminary nature on the production of pigment by mycobacteria are presented in table 1. The pigment was produced on Youmans medium but not on Dubos Tween-albumin medium. As the major difference between the two is that the former is very rich in carbohydrate (glycerol), it was thought that the carbon source was primarily responsible for the effect. Addition of either glucose, glycerol, or *meso*-inositol to Dubos medium confirmed the supposition.

In order to gain a greater insight into the biochemical aspects of the formation of the pigment, the effect of various carbohydrates, nitrogen sources, and purines and pyrimidines was studied. The results of studies with carbohydrates are given in table 2. While several carbohydrates supported growth, only a few enabled the pro-

TABLE 3

EFFECT OF PURINES AND PYRIMIDINES ON PIGMENT PRODUCTION BY MYCOBACTERIA

| Strain | Control | Increase in Pigment Production over Control with: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Adenine | Adenosine | Hypoxanthine | Inosine | Guanine | Guanosine | Xanthine | Xanthosine | Thymine | Uracil | Amino-imidazole-carboxamide |
| *M. tuberculosis* H37Rv | − | − | − | − | − | − | − | − | − | − | − | − |
| *M. tuberculosis* H37Rv-AmR | ++ | − | − | − | − | − | − | − | − | − | − | − |
| *M. tuberculosis* Ravenel | − | − | − | − | − | − | − | − | − | − | − | − |
| *M. tuberculosis* Ravenel-AmR | − | − | − | − | − | − | − | − | − | − | − | − |
| *M. tuberculosis* Sheard | − | − | − | − | − | − | − | − | − | − | − | − |
| *M. smegmatis* (saprophyte) | +++ | ++ | ++ | +++ | +++ | − | − | − | − | − | − | + |
| *M. butyricum* (saprophyte) | ++++ | ++ | ++ | +++ | +++ | − | − | − | − | − | − | + |
| *M. lacticola* (saprophyte) | +++ | ++ | ++ | +++ | +++ | − | − | − | − | − | − | + |

− = No pigment; + to ++++ = increasing amounts of pigmentation.

TABLE 4

EFFECT OF FLAVIN-ADENINE-DINUCLEOTIDE (FAD) AND CULTURE FILTRATES OF MYCOBACTERIA ON KIDNEY D-AMINO ACID OXIDASE

| Time | Oxygen Uptake in Microliters* | | | | | |
|---|---|---|---|---|---|---|
| | Control | FAD 2.5 γ ml. | H37Rv Filtrate | H37Rv-AmR Filtrate | *M. Smegmatis* Filtrate | *M. Lacticola* Filtrate |
| (*minutes*) | | | | | | |
| 30 | 11.0 | 36.5 | 11.0 | 9.0 | 7.6 | 8.6 |
| 60 | 22.1 | 66.0 | 20.1 | 18.9 | 18.5 | 21.9 |
| 90 | 30.4 | 85.7 | 27.5 | 25.9 | 28.2 | 31.5 |

* Each Warburg vessel contained:

| | |
|---|---|
| Kidney D-amino acid oxidase (0.8 mg.) | = 1.0 ml. |
| FAD (5γ) or culture filtrate | = 0.8 ml. |
| DL-alanine (substrate) 2 mg. | = 0.2 ml. |
| pH reaction mixture | = 8.9 |
| KOH (15 per cent) (center well) | = 0.2 ml. |

duction of pigment, showing thereby that the two functions were independent of each other. Little growth occurred with *meso*-inositol, but production of pigment was maximal and assumed a reddish color. With all other carbon sources, the color of the pigment was yellow.

The following nitrogen sources were incorporated at 0.5 per cent concentration omitting asparagin from the medium: sodium nitrite, potassium nitrate, ammonium sulfate, urea, asparagin, 1 (+) lysine, beta-alanine, DL-norvaline, DL-norleucine, DL-phenylalanine, DL-methionine, 1 (−) leucine, DL-serine, glycine, DL-threonine, DL-valine, 1 (+) arginine, 1 (−)histidine, 1 (−) cystine, 1 (+) tyrosine, 1 (+) glutamic acid, and DL-alanine. It was observed that histidine alone stimulated pigment synthesis especially by H37Rv-AmR.

The results are presented in table 3 of the effect of purines, their derivatives, and pyrimidines on pigment formation by mycobacteria on Youmans medium containing 1 per cent inositol added in addition to the glycerol. It was observed that hypoxanthine and adenine, and their derivatives, and 4-amino-5-imidazole-carboxamide stimulated pigment synthesis by saprophytes, while no such effect was demonstrable with tubercle bacilli. The other substances investigated did not show any effect.

TABLE 5

EFFECT OF PURINES AND THEIR DERIVATIVES ON GROWTH INHIBITION OF MYCOBACTERIA BY 6-MERCAPTOPURINE

| Strain | Control | 6-Mercaptopurine* with: | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No Addition | Adenine | Adenosine | Adenosine-monophospate | Yeast Adenylic Acid | Hypoxanthine | Inosine | Inosinic Acid | Guanine | Guanosine | Xanthine | Xanthosine |
| *M. tuberculosis* H37Rv | +++ | − | − | − | + | ++ | +++ | +++ | +++ | ++ | ++ | − | − |
| *M. tuberculosis* H37Rv-AmR | +++ | ± | − | − | ++ | ++ | +++ | +++ | +++ | ++ | ++ | − | − |
| *M. tuberculosis* Ravenel | +++ | − | − | − | + | + | +++ | +++ | +++ | ++ | ++ | − | − |
| *M. tuberculosis* Ravenel-AmR | +++ | − | − | − | + | ++ | +++ | +++ | +++ | ++ | ++ | − | − |
| *M. tuberculosis* Sheard | +++ | + | + | + | ++ | ++ | +++ | +++ | +++ | ++ | ++ | − | − |
| *M. smegmatis* (saprophyte) | ++++ | + | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| *M. butyricum* (saprophyte) | ++++ | + | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| *M. lacticola* (saprophyte) | ++++ | + | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |

* 6-Mercaptopurine concentration in medium for tubercle bacilli, 10γ per ml.; and for saprophytes, 100 γ per ml.

TABLE 6

DEAMINASES OF ADENINE AND ADENOSINE IN MYCOBACTERIA

| Organism | Adenine Deaminase | Adenosine Deaminase |
|---|---|---|
| *M. tuberculosis* H37Rv | − | − |
| *M. tuberculosis* H37Rv-AmR | − | − |
| *M. tuberculosis* Ravenel | − | − |
| *M. tuberculosis* Ravenel-AmR | − | − |
| *M. tuberculosis* Sheard | − | − |
| *M. smegmatis* (saprophyte) | − | ++ |
| *M. butyricum* (saprophyte) | − | +++ |
| *M. lacticola* (saprophyte) | − | +++ |

− = No activity; + to +++ = increasing activity.

Examination for the presence of flavin-adenine-dinucleotide (FAD) in the pigmented culture filtrates by the Warburg technique using kidney *d*-amino acid oxidase showed that none of the filtrates could stimulate the enzyme as FAD did (table 4).

The results of "inhibition analysis" studies with 6-mercaptopurine as the inhibitory analogue using several of the purines and their derivatives are presented in table 5. 6-Mercaptopurine inhibited the growth of tubercle bacilli between 1 and 10 gamma per ml., while saprophytes were only partially inhibited at 100 gamma per ml. All purines and their derivatives reversed the inhibitory effect with saprophytes. With tubercle bacilli, adenine, adenosine, xanthine, and xanthosine were without effect. While hypoxanthine or its derivatives counteracted 6-mercaptopurine best, followed by guanine and guanosine, yeast adenylic acid and adenosine monophosphate type I were only partially effective.

Tests for the presence of nucleodeaminases showed that saprophytes had adenosine deaminase, while with tubercle bacilli the deaminases of both adenine and adenosine were not detectable (table 6).

## DISCUSSION

From the results presented in table 1, it may be noted that pigment production was intimately associated with the presence of a carbon source in the medium. Addition of either glucose, glycerol, or *meso*-inositol greatly stimulated the synthesis of the pigment in Dubos medium, which by itself did not support pigmentation.

Since H37Rv-AmR produced the pigment, while Ravenel-AmR and Sheard did not show such a coloration, it was first presumed that pigment production by amithiozone-resistant mutants was probably an exclusive property of human tubercle bacilli. However, subsequent examination of several strains of tubercle bacilli of human origin made resistant to amithiozone revealed that the encountered mutant, H37Rv-AmR, was indeed unique in this respect.

In order to identify the immediate carbohydrate precursor, a number of sugars were tested (*see* table 2). Among these, *meso*-inositol was the best for pigment production by amithiozone-resistant saprophytes and the mutant H37Rv-AmR. In the presence of *meso*-inositol, the yellow color of the pigment, as seen with other carbohydrates such as glycerol, changed to reddish-yellow. The part played by inositol in this case is analogous to its stimulation of riboflavin formation by *Eremothecium ashbyii* (6). Inositol probably contributes the D-ribose moiety following oxidation to *allo*-mucic acid (7), which has the same configuration as D-ribose.

Tests with different nitrogenous substances including nitrite, nitrate, ammonium salts, amides, and amino acids revealed that histidine alone stimulated pigment synthesis especially by H37Rv-AmR. Histidine has been shown to be one of the precursors in riboflavin synthesis, and it was presumed that the imidazole ring was incorporated into the *iso*-alloxazine nucleus of the vitamin (8).

A number of purines, their derivatives, and pyrimidines have been tested. The results showed that adenine, adenosine, hypoxanthine, and 4-amino-5-imidazole-carboxamide greatly stimulated pigmentation with saprophytes on a medium containing added inositol, while no such significant effect could be demonstrated with H37Rv-AmR, Ravenel-AmR, and Sheard. Xanthine, xanthosine, guanine, guanosine, uracil, and thymine were without effect (*see* table 3).

As *meso*-inositol, histidine, and the purines were shown to be involved in the biosynthesis of riboflavin (6, 8, 9), the stimulatory effect on pigment production obtained with these compounds pointed toward the formation of a flavin-nucleotide. However, fluorimetric analysis of the pigmented culture filtrates did not indicate the presence of any significant amount of the vitamin. The extract showed a blue fluorescence which was not affected by dithionite. Determination of flavin-adenine-dinucleotide by the D-amino

acid oxidase test using the Warburg apparatus also demonstrated the absence of any coenzyme in the boiled or partially hydrolyzed culture filtrates (*see* table 4). These studies, therefore, ruled out any association of the pigment with riboflavin.

The pigment also did not correspond to any of those described in literature pertaining to tubercle bacilli (10). However, it is known that pigment production by mutant strains alone has been concerned either with metabolic deficiencies, as with the ascomycete fungus Neurospora relating to nicotinic acid (11) and adenine (12); or with resistance to one agent or another, such as phage resistance in *Salmonella typhi* (13), and sulfanilamide resistance in *Mycobacterium ranae* (14). It was observed that the sulfanilamide-resistant *M. ranae* elaborated a diazotizable aromatic amine in the culture fluid, and this is similar to the accumulation of 4-amino-5-imidazole-carboxamide by sulfadiazine-inhibited cells of *E. coli* in the absence of methionine (15). It is established that sulfanilamide interferes with *p*-aminobenzoic acid, which is involved in the synthesis of methionine. Methionine contributes the methyl group for carbon-2 in the conversion of the amine to hypoxanthine.

In the present study, spectrophotometric observation of the pigmented culture filtrates showed a peak absorption around 265 mμ and nothing at 375 mμ and 450 mμ, again demonstrating the absence of any riboflavin, as all riboflavin compounds show intense absorption at these three wavelengths (16). The absorption at 265 mμ indicated the probable involvement of a purine substance and, since the purines stimulated the synthesis of the pigment, the relationship of amithiozone resistance to alteration in purine metabolism of tubercle bacilli was considered.

Mitchell and Houlahan (12) had demonstrated that some of the adenine-requiring mutants of Neurospora produced strong pigmentation in the culture fluid, which slowly accumulated in the mycelium as the age of the culture increased. The chemical nature of the pigment was not easy to analyze. Mitchell (17) indicated that it was a polymerization product of an amino-imidazole-ribotide together with a related purine derivative, and that there was a great variation in the size of the polymer; the latter could eventually account for the differences in color. The possibility was considered that amithiozone resistance involves a genetic block in the purine metabolism of tubercle bacilli as well, and that the pigment produced by these organisms was analogous to that produced by Neurospora mutants. This would explain why *meso*-inositol, histidine, and the purines stimulated the formation of the pigment in mycobacteria. It further implied that amithiozone affects in some way the purine synthesis of the susceptible tubercle bacilli. A block in such a synthesis with tubercle bacilli does not result in any nutritional deficiency for the purine as with the mutants of Neurospora. Another mechanism for its biosynthesis must, therefore, exist in tubercle bacilli since adenine is most essential for nucleic acid formation and reproduction. The ability to convert guanine to adenine, or vice versa, is known to exist in several microorganisms, and such conversions take place at the polynucleotide level (18).

On the assumption that mutation to amithiozone resistance involves a genetic block in the pathway of adenine synthesis via hypoxanthine, the biosynthetic mechanism was next studied. Tubercle bacilli, susceptible or resistant to amithiozone, do not manifest any exogenous requirement for adenine or hypoxanthine, and they are able to grow well in synthetic media. Adenine and hypoxanthine exist in several different combinations in living systems,—the purine, the riboside, the ribotide and nucleotide, and the coenzyme—and the problem as to which of these derivatives are utilizable by tubercle bacilli and which are not is indeed a very difficult one. To an extent, this difficulty was overcome by the use of the technique of "inhibition analysis." It was presumed that, if a metabolite is able to counteract the growth-inhibitory effects of an antagonist, the bacteria have the particular enzyme system adapted to the utilization of the specific metabolite. Among the several purine antagonists investigated, it was observed that 6-mercaptopurine showed the maximal inhibitory activity toward tubercle bacilli and that 2, 6-diaminopurine was less active. In presence of sub-inhibitory concentrations of 2, 6-diaminopurine, *M. butyricum* and *M. lacticola* produced greater amounts of the pigment with added adenine, hypoxanthine, and their derivatives. This further demonstrated that the pigment in question is a product of a block in the metabolism of the purines.

Reversal studies with 6-mercaptopurine as the antimetabolite revealed that all mycobac-

teria, whether or not resistant to amithiozone, utilized guanine, hypoxanthine, and their derivatives, to counteract the inhibition (*see* table 5). Xanthine and xanthosine were active for the saprophytes alone. While the saprophytes utilized adenine and its derivatives with equal facility, tubercle bacilli were unable to assimilate either adenine or adenosine, while yeast adenylic acid and adenosine monophosphate type I were partially effective. This inability of tubercle bacilli to use adenine or adenosine, hypoxanthine being active, is due to the lack of adenine- and adenosine-deaminases in these organisms, while the saprophytes have adenosine-deaminase (*see* table 6). Adenine is probably converted to adenosine by saprophytes by transglycosidation. These studies clearly emphasize that the synthesis of adenine by tubercle bacilli proceeds only at the ribotide or nucleotide level, while with saprophytes such a function may be accomplished even at the free purine or ribotide stage. Further, as both amithiozone-susceptible and -resistant tubercle bacilli were able to utilize hypoxanthine and its derivatives, which are the first in the pathway to purine metabolism in biologic systems, the location of the genetic block in amithiozone-resistant mutants must lie before the synthesis of hypoxanthine itself (figure 1).

Vischer and associates (19) demonstrated that the amount of nucleic acid guanine in tubercle bacilli of avian origin is high. While the ratio of adenine to guanine in these organisms was 1:2.3, with tubercle bacilli of human and bovine origin it was found to be 1:1.6 (20). It must be pointed out that tubercle bacilli of avian origin are naturally resistant to amithiozone, and they presumably form the bulk of their purine requirement for nucleic acid synthesis through guanine. Amithiozone-susceptible tubercle bacilli of human and bovine origin direct their nucleic acid purine synthesis in addition through hypoxanthine and adenine, and that a block in this synthesis may give rise (as with Neurospora) to pigmented and nonpigmented resistant mutants, according to the position of the geneblock, is outlined in figure 2. The inability of either hypoxanthine, adenine, or their derivatives, to stimulate pigment synthesis by H37Rv-AmR would be consistent with the location of the genetic block at the labile purine precursor-hypoxanthine stage. With the saprophytes the utilization of methionine for hypoxanthine synthesis is blocked; these strains even normally produce the pigment, which is greatly stimulated by *meso*-inositol, histidine, and the purines. Additional evidence for the defect in methionine metabolism in saprophytes and in tubercle bacilli of avian origin is provided by the fact that para-aminosalicylic acid, which has been shown to be involved in methionine synthesis (21), is without action on these organisms. Tubercle bacilli of avian origin are resistant to amithiozone because they do not synthesize nucleic acid purines using methionine, and probably divert the pathway through guanine. The other nonpigment-producing amithiozone-resistant mutants of tubercle bacilli of human and bovine origin are eventually blocked in the synthesis of the labile purine precursor itself.

One of the possible sites for amithiozone action is interference with the metabolism of methionine. Methionine is known to contribute the methyl group, converted to "active formate," for the carbon-2 of the purine ring (15). Methionine may be converted to "active methionine" (adenosyl-methionine) as a preliminary step in this process (22). Amithiozone eventually enters in place of methionine nonreversibly to yield nucleic

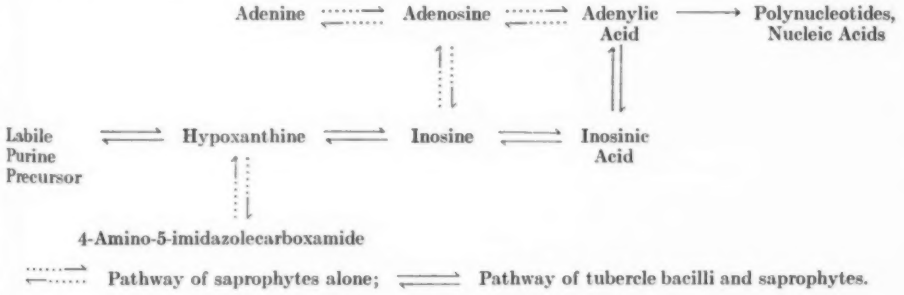


FIG. 1. Metabolism of hypoxanthine and adenine by mycobacteria.

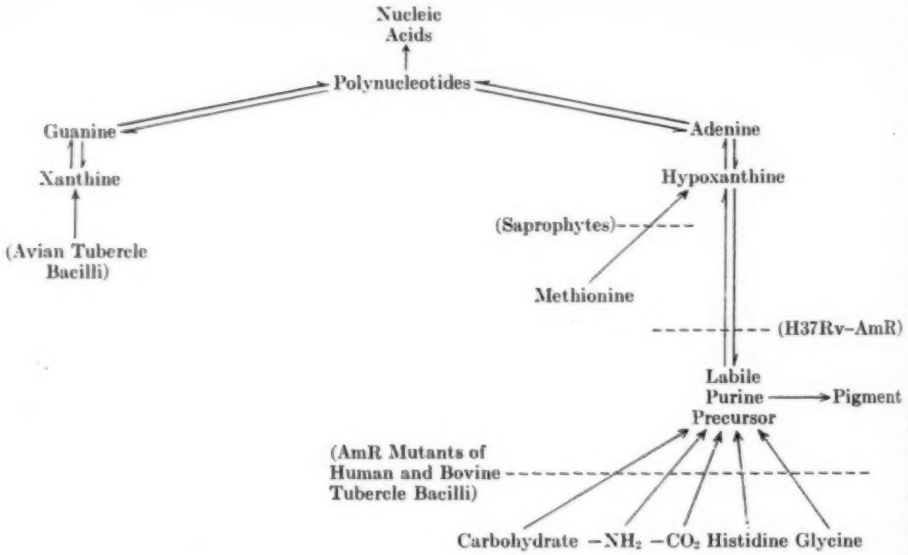


FIG. 2. Purine metabolism in relation to amithiozone resistance in mycobacteria.

acid fractions that would not be capable of reproduction. This view is supported by the fact that methionine could correct the hepatic dysfunction caused by thiosemicarbazones (23). The ability of thiosemicarbazones to inhibit the multiplication of viruses (24) and leukemic cells (25), and of thiouracil to enter into the composition of nucleic acids (26) reported in the literature, gives additional evidence that amithiozone-like substances interfere with nucleic acid metabolism. The cross-resistance of various thiourea derivatives with thiosemicarbazones may be accomplished at this site.

The conclusions that can be drawn from the above are that, in order to be susceptible to amithiozone, and thiosemicarbazones in general, the organisms must possess the enzyme system necessary for the assimilation of methionine for hypoxanthine synthesis and that any genetic mutation that would lose one enzyme or another in this synthetic series would render the organism resistant to the action of the drug.

The peculiar possession of certain enzyme systems makes organisms susceptible to certain drugs; loss of the enzyme enables them to be resistant, and such a loss is accomplished by mutation. In an earlier investigation with isoniazid by the writers (2), it was shown that possession of cellular peroxidase made the tubercle bacilli highly susceptible to isoniazid, and loss of this enzyme by mutation rendered them resistant to a great extent. With amithiozone, loss of an enzyme in purine synthesis rendered the strain resistant to the drug. The appearance of a pigment, which is an acquired characteristic, is in reality the result of a loss of function in the utilization of the precursor for hypoxanthine synthesis. Likewise, it is known that loss of existing receptors makes bacteria resistant to the action of phage. Similar observations have been made with colicins (27) and animal viruses. Consideration of these facts would suggest that an important mechanism in the development of resistance is simply the loss of a susceptible reaction. Since mutations almost always involve loss of function, such a view would well conform to accepted principles of genetics.

## SUMMARY

An amithiozone-resistant mutant, H37Rv-AmR, produced a yellow pigment in the culture fluid similar to that produced by the naturally resistant saprophytes. The results of studies on the biosynthesis of the pigment by both H37Rv-

AmR and the saprophytes showed a striking parallel requirement; *meso*-inositol and histidine stimulated pigment formation in all strains, while hypoxanthine and adenine were active only for the saprophytes.

The pigment so produced was not a flavine or any of those previously described in the literature on mycobacteria; it is thought to be a polymerization product of an amino-imidazole-ribotide together with a related purine derivative, similar to the pigment described by Mitchell (17) as occurring with adenine-requiring mutants of Neurospora.

Amithiozone enters purine metabolism of susceptible tubercle bacilli which utilize methionine for nucleic acid synthesis, giving rise to fractions not capable of reproduction.

Loss of a mechanism involving the biosynthesis of hypoxanthine using methionine yields amithiozone-resistant mutants. The locations of the genetic blocks are at different places with different strains, which explains the evolution of pigmented- and nonpigmented-resistant mutants.

The possession of an enzyme by tubercle bacilli of human and bovine origin, in addition to those found in tubercle bacilli of avian origin and the saprophytes, makes them susceptible to amithiozone. Any mutation affecting this pathway, resulting in a loss of one or more enzymes, leads to resistance.

It is proposed that the loss of a susceptible pathway is much more likely to be responsible for the development of resistance to drugs than the acquisition of a protective mechanism, a view which would better conform to the genetic knowledge.


## Sumario

*La Amitiozona: Mecanismo de su Acción y Formación de Resistencia por las Micobacterias*

Una mutante amitiozonorresistente, H37Rv-AmR, produjo un pigmento amarillo en el líquido de cultivo, lo mismo que los saprofitos resistentes naturalmente. Los resultados de los estudios relativos a la biosíntesis del pigmento tanto por la H37Rv-AmR como por los saprofitos revelaron un notable requisito paralelo: el *meso*-inositol y la histidina excitaron la pigmentogenia en todas las cepas, mientras que la hipoxantina y la adenina no se mostraron activas más que para los saprofitos.

El pigmento producido en dicha forma no era una flavina ni ninguno de los pigmentos ya descritos en la literatura referente a las micobacterias; parece ser un producto de la polimerización de un amino-imidazol-ribótido junto con un derivado purínico afín, semejante al pigmento descrito por Mitchell (17) como coexistente con las mutantes de las Neurospora que requieren adenina.

La amitiozona interviene en el metabolismo de la purina por los bacilos tuberculosos susceptibles que utilizan metionina para la síntesis del ácido nucleico, produciendo fracciones que son incapaces de reproducirse.

La pérdida de un mecanismo que interviene en la biosíntesis de la hipoxantina usando metionina rinde mutantes amitiozonorresistentes. La localización de los bloqueos genéticos radica en distintos sitios en distintas razas, lo cual explica la evolución de mutantes resistentes pigmentadas y sin pigmentación.

La posesión de una enzima por los bacilos tuberculosos de procedencia humana y bovina, además de las descubiertas en los bacilos tuberculosos de procedencia aviaria y en los saprofitos los susceptibiliza a la amitiozona. Toda mutación que afecte dicha vía, ocasionando la pérdida de una o más enzimas, conduce a la formación de resistencia.

Se alega que es mucho más probable que la pérdida de una vía susceptible, y no la adquisición de un mecanismo protector, sea la causa de la formación de resistencia a las drogas, opinión esta que se conforma mejor a los conocimientos genéticos.



## Resume

*Amithiozone: mécanisme d'action et développement de la résistance par les mycobactéries*

Un mutant amithiozone-résistant, H37Rv-AmR, a produit un pigment jaune dans le liquide de culture, comme les saprophytes naturellement résistants. Les résultats des recherches sur la biosynthèse du pigment par H37Rv-AmR et par les saprophytes montraient que les conditions requises étaient singulièrement semblables; le *méso*-inositol et l'histidine entraînaient la formation de pigment dans toutes les souches, tandis que l'activité de l'hypoxantine et l'adénine ne s'exerçait que sur les saprophytes.

Le pigment ainsi produit n'était ni une flavine ni aucune de ceux précédemment décrits dans la littérature sur les mycobactéries; les auteurs estiment qu'il s'agit d'un produit de polymérisation d'un amino-imidazol-ribotide associé a un dérivé purine apparenté, semblable au pigment décrit par Mitchell (17) tel qu'il se produit avec les mutants de neurospores réclamant l'adénine.

L'amithiozone entre dans le métabolisme de la purine des bacilles tuberculeux sensibles utilisant la methionine pour la synthèse de l'acide nucléique, en donnant naissance à des fractions incapables de reproduction.

La perte d'un mécanisme comportant la biosynthèse de l'hypoxantine à l'aide de la méthionine produit des mutants amithiozone-résistants. Les blocs génétiques siègent en différents points selon les différentes souches, ce qui explique le développement de mutants résistants pigmentés et non-pigmentés.

Le fait que les bacilles tuberculeux d'origine humaine ou bovine possèdent un enzyme en plus de ceux trouvés dans les bacilles tuberculeux d'origine aviaire et dans les saprophytes, les rend sensibles à l'amithiozone. Toute mutation affectant ce processus métabolique et se traduisant par la perte de l'un ou de plusieurs enzymes conduit à la résistance.

Les auteurs suggèrent que la perte éventuelle d'un processus métabolique est plus vraisemblablement responsable du développement de la résistance au médicament que l'acquisition d'un mécanisme de protection, conception qui se conformerait mieux aux donneés de la génétique.

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# THE TUBERCULIN REACTIONS OF 530 PATIENTS ADMITTED TO THE TUBERCULOSIS SERVICE, FITZSIMONS ARMY HOSPITAL[1]

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## INTRODUCTION

The tuberculin testing results have been analyzed in 530 patients with tuberculosis or possible tuberculosis who were admitted to the Tuberculosis Male Admitting Ward, Fitzsimons Army Hospital, during the years 1956–1958. This review was carried out in an effort to answer several problems:

To determine if a completely negative reaction to a tuberculin test employing second-strength PPD is a valid test for exclusion of tuberculosis in other than cases of very early infection or in moribund patients.

To determine whether a negative reaction to intermediate-strength PPD is a reliable indication of freedom from active tuberculous disease in the individual case.

To determine the significance of second-strength PPD in patients who are clinically suspected to have active tuberculosis but in whom intermediate-strength PPD is negative.

To compare the validity of forty-eight-hour readings versus seventy-two-hour readings.

To determine the incidence of false negative reactions to tests, if any.

## MATERIALS AND METHODS

All of the patients were tested with commercially available skin test antigen (PPD) in first strength (0.00002 mg.). If their reactions were negative, intermediate-strength PPD (0.0001 mg.) was applied, followed by second-strength PPD (0.005 mg.) if reactions to the intermediate-strength test were negative. All of the patients were simultaneously tested with histoplasmin and coccidioidin 1:100. All of the tests were applied and read by a physician on the admitting service. All of the tests were read at forty-eight and seventy-two hours, and the results were recorded in both millimeters of erythema and of induration. Commercially available antigens were deliberately used in this study since these are in most common general usage throughout the country.

## OBSERVATIONS

The classification related to the test level and millimeters of induration in the 530 patients is shown in table 1. As would be expected on a service where the bulk of admissions are for active tuberculosis, the great majority of the patients reacted strongly on initial testing with PPD of first- or intermediate strength. In the 3 patients who were nonreactors on the first testing and gave definitely positive results on repeat testing, the results are probably ascribable to errors in technique. Three patients whose reactions to PPD showed erythema but no definite induration were found to be definite reactors with induration of 10 mm. or more on re-testing with Old Tuberculin 1:1,000. Two of these patients had active pulmonary tuberculosis, one with pathologic confirmation following surgery. In the third case, no evidence of pulmonary disease was confirmed. All 27 patients with repeated and unequivocally negative tuberculin reactions eventually were considered to be free of tuberculosis. In 5 patients no final classification was possible because of confusing skin test results.

The results in 492 patients whose tuberculin reactions were definitely positive in forty-eight hours by size of induration and strength of tuberculin reagent are analyzed in table 2. Over three-fourths showed reactions of 10 mm. or more to one of the three dilutions used. Another 20 per cent showed only a 5–9 mm. induration to either first- or intermediate-strength PPD. Twelve patients, or 2.5 per cent of the reactors, showed only a 5–9 mm. induration to second-strength PPD. In 4 of these patients, the histoplasmin reaction was positive and in one, the coccidioidin reaction was positive. Therefore, the possibility of a cross-reaction arises in these cases. However, great care has been taken on the service to isolate syringes used for one type of testing from those used for other skin tests, which makes this quite a remote possibility.

As will be noted from table 3, 11 of these 12 patients with only weak reactions to second-strength PPD were eventually considered to be free from any evidence of active tuberculous disease. One patient was definitely demonstrated

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TABLE 1

THE REACTIONS TO TUBERCULIN OF 530 PATIENTS ON THE TUBERCULOSIS ADMITTING WARD, FITZSIMONS ARMY HOSPITAL

| Test Level and Reactions | Mm. of Induration at 48 Hours | | | Total | Per Cent |
|---|---|---|---|---|---|
| | 0–4 | 5–9 | 10 and Greater | | |
| Positive to PPD #1* (initial test) | | 82 | 302 | 384 | 72.4 |
| Positive to PPD #1½† (initial test) | | 4 | 22 | 26 | 4.9 |
| Negative to PPD #1; positive to PPD #1½ | | 12 | 17 | 29 | 5.5 |
| Negative to PPD #1; positive to PPD #2‡ | | 1 | 6 | 7 | 1.3 |
| Negative to PPD #1 and #1½; positive to PPD #2 | | 11 | 32 | 43 | 8.1 |
| Initially negative to PPD #2; positive in repeat test | | | 3 | 3 | 0.6 |
| Negative at 48 hours | | 2 | 1 | 3 | 0.6 |
| Positive at 72 hours | | | 3 | 3 | 0.6 |
| Negative or (?) to PPD; positive to OT | | | | | |
| Definitely negative to PPD #2 | 27 | | | 27 | 5.1 |
| No final classification possible | — | — | — | 5 | 0.9 |
| Total number | 27 | 112 | 386 | 530 | |
| Total per cent | 5.1 | 21.1 | 72.9 | | 100 |

* PPD #1 = first-strength PPD.
† PPD #1½ = intermediate-strength PPD.
‡ PPD #2 = second-strength PPD.

to have very far advanced active cavitary tuberculosis with tubercle bacilli in the sputum.

The high proportion ultimately found to be free from clinically active tuberculosis would indicate that a weakly positive reaction to second-strength PPD should be interpreted with caution as it might be either a nonspecific reaction or the very low level of reactivity that is occasionally seen with active disease. However, undoubted clinically active tuberculosis may be present with a very weakly positive tuberculin reaction and, therefore, reactions of this nature cannot be entirely disregarded.

The percentage of reactions that will be detected by use of intermediate-strength PPD are shown in table 4. In the first column, the conventional criteria are used designating as positive any test with a reaction of 5 mm. or more at any dilution. Using this criteria, it may be seen that 8.8 per cent will react negatively at the intermediate-strength level, but will be positive at the second-strength level. As mentioned in the preceding paragraph, weakly positive second-strength results must be viewed with a certain doubt. In the second column, the definition of a positive reaction is modified by eliminating all second-strength PPD reactions showing less than 10 mm. induration. There thus remain 6.7 per cent of positive reactions which will be missed if only the intermediate level is used.

If all second-strength reactions of less than 10 mm. are excluded as being of dubious significance, one would conclude that the intermediate-strength test should detect nearly 95 per cent of tuberculin reactors and that, as a screening procedure in which only one test can be used (as in mass surveys), intermediate-strength PPD approaches an ideal reagent. If it be assumed, as indicated in studies of the U. S. Public Health Service, that some of the reactions of 10 mm. or more to PPD of the second strength might also be nonspecific in nature, the validity of using the intermediate-strength reagent in screening procedures is further enhanced.

It now remains to evaluate the significance of a strongly positive reaction to second-strength PPD in the face of a negative reaction to the intermediate strength in a patient suspected to have active tuberculosis.

In table 5 the final diagnosis is shown in the 32 patients with negative reactions to intermediate-strength PPD and a 10 mm. or greater reaction to the second-strength test. It is to be

TABLE 2

SIZE OF REACTION AND STRENGTH OF REAGENT IN 492 PATIENTS WITH 5 MM. OR MORE INDURATION TO PPD AT 48-HOUR READING

| Mm. of Induration and Strength of PPD | Number | Per Cent |
|---|---|---|
| 10 mm. or more to PPD #1, 1½, or 2* | 382 | 77.5 |
| 5–9 mm. to PPD #1 or 1½ | 98 | 20.0 |
| 5–9 mm. to PPD #2 | 12 | 2.5 |
| Total | 492 | 100.0 |

* See legends to Table 1.

remembered that the group represents nearly 7 per cent of the positive reactors. Twenty-three of these patients were finally diagnosed as having active tuberculosis. Fifteen of the 23 had their diagnosis proved either by bacteriologic or biopsy findings. Nine of these patients were eventually considered to have no evidence of active tuberculosis. It could be assumed that the results in all of these 9 patients represented nonspecific false positive reactions, and that some of the 8 presumptive tuberculosis diagnoses were also erroneous. Nevertheless, approximately two-thirds of the 10 mm. or greater reactions to second-strength tuberculin in the face of a negative reaction to the intermediate strength would have been valid positive reactions, as evidenced by the presence of clinically active tuberculosis.

Accordingly, it must be concluded that a negative reaction to intermediate-strength PPD cannot be used as an exclusion test in the individual patient with a suspicious lesion, and that a reaction to the second-strength PPD of 10 mm. or more induration should be clinically regarded as a valid positive test even though the possibility that this may be merely a strong nonspecific reaction is conceded.

The changes noted in comparison readings at forty-eight or seventy-two hours are summarized in tables 6 and 7. It will be noted that in more than 85 per cent of the cases there was agreement at both readings. However, in nearly 12 per cent there was a significant decrease in the degree of reaction between the forty-eight- and seventy-two-hour readings, although approximately 3 per cent will show a significant increase.

It is perhaps more noteworthy that in 22 patients (or 4.4 per cent of the positive tests) the reactions have been read as positive at the forty-eight-hour reading but as negative after seventy-two hours, whereas only 3 (or 0.6 per cent) would be read as negative at forty-eight and positive at seventy-two hours.

It thus appears that, whenever possible, readings should be made at both forty-eight and seventy-two hours and that the most positive of the two be accepted as the final reading. However, in situations in which only one reading is feasible, it is preferable to read the forty-eight-hour result.

TABLE 3

FINAL DIAGNOSIS OF 12 PATIENTS WITH 5-9-MM. REACTIONS TO SECOND-STRENGTH PPD

| | |
|---|---|
| Nontuberculous or no disease found | 11 |
| Proved to have far advanced cavitary tuberculosis with infectious sputum | 1 |

TABLE 4

THE REACTION TO INTERMEDIATE-STRENGTH PPD AS A SCREENING TEST

| | Positive to PPD #2 at 5 Mm. or More | Positive to PPD #2 at 10 Mm. or More |
|---|---|---|
| Negative to PPD #1½; positive to PPD #2* | 43 | 32 |
| Total positive to PPD at 48 hours | 492 | 480 |
| Total per cent negative to PPD #1½ and positive to PPD #2 | 8.8 | 6.7 |

* See legends to Table 1.

In 5 patients, classification on the basis of tuberculin testing was impossible. One patient in this group had proved pulmonary tuberculosis, but the tuberculin testing was recorded variously in different parts of the record. The confusion here is probably clerical, and the patient can be eliminated from further consideration.

The second patient showed a questionable 5-mm. induration after seventy-two hours on one examination, and a very questionable infiltration on repeat examination with second-strength PPD; the patient was not tested with Old Tuberculin. Clinically, by roentgenographic examination and by lymph node biopsy, this patient was considered to have sarcoidosis and was eventually transferred to the Veterans Administration with this diagnosis.

The third patient was completely negative to intermediate-strength PPD, as well as to histoplasmin and coccidioidin. His reaction to second-strength PPD on two occasions showed considerable erythema of 20 to 40 mm.; however, induration was not definite and, if present at all, was exceedingly ill defined. Unfortunately, recheck testing with OT was not done in this patient. Clinically, this patient had a resolving bacterial pneumonia and was eventually considered to be nontuberculous and returned to duty.

The fourth patient originally showed a definitely positive reaction to PPD of the first strength. However, he had a highly positive

TABLE 5

ANALYSIS BY FINAL DIAGNOSIS OF 32 PATIENTS WITH NEGATIVE REACTIONS TO INTERMEDIATE-STRENGTH PPD AND 10 MM. OR MORE INDURATION REACTIONS TO SECOND-STRENGTH PPD

| | | |
|---|---|---|
| Final Diagnosis: | | |
| Active tuberculosis | | 23 |
| Proved tuberculous by bacteriologic findings or biopsy | 14 | |
| Pulmonary tuberculosis: | | |
| Minimal | 1 | |
| Moderately advanced | 5 | |
| Far advanced | 2 | |
| Pleurisy | 5 | |
| Pericarditis | 1 | |
| Presumptive Diagnosis (clinical; negative bacteriologic findings; no biopsy) | 8 | |
| Pulmonary tuberculosis | 4 | |
| Pleurisy | 3 | |
| Pericarditis | 1 | |
| Proved chromogenic mycobacteria | 1 | |
| Final nontuberculous diagnosis | | 9 |
| Presumption of tuberculosis dropped as result of surgery | 3 | |
| Nonspecific pleuritis | 2 | |
| Nonspecific pericarditis | 1 | |
| Cirrhosis of liver with pleural effusion | 2 | |
| Pulmonary fibrosis | 1 | |
| Coccidioidomycosis | 1 | |
| Pneumonia, etiology undetermined | 1 | |
| Spontaneous pneumothorax | 1 | |

histoplasmin skin test reaction, and repeat testing using a new syringe showed a definitely negative reaction to first-strength PPD. This undoubtedly is an example of cross-contamination of syringes in a patient who was very strongly sensitive to histoplasmin. Intermediate-strength PPD was definitely negative. The reaction to second-strength PPD showed an exceedingly ill-defined induration which was read by numerous observers, some of whom called it negative, others dubious, and still others, probably positive. Unfortunately, no skin testing with OT was performed. The patient had a right upper lobe infiltration which looked like tuberculosis on the roentgenogram. One gastric culture yielded typical tubercle bacilli. Furthermore, his disease responded well to the usual tuberculosis therapeutic regimen. In this case, the dubious reaction to PPD should have led to further testing with OT, which possibly would have resolved the doubt.

The final patient is of considerable interest in that numerous PPD and OT reactions were studied, all of which yielded considerable erythema but no definite induration. However, following each injection, a brownish discoloration developed at the site of injection and persisted for several weeks. The patient's histoplasmin reaction was positive and the reaction to coccidiodin was negative. The patient was considered clinically to have histoplasmosis with a probable cross-reaction. The pathologic findings were those of caseous tuberculosis. Smears from the resected specimen, however, showed atypical beaded chains of acid-fast bacilli, and culture of the same material yielded chromogenic mycobacteria. This patient made a good clinical recovery on the usual tuberculosis therapeutic regimen. His case is presented as an example of tuberculosis due to atypical organisms with an atypical skin test response.

It should be noted that no patient in this series with an unequivocally negative reaction to PPD subsequently was shown to have clinical tuberculosis. However, several patients, all of whom had relatively large areas of erythema with indefinite or ill-defined induration, were shown to have active tuberculosis. In these cases,

TABLE 6

COMPARISON OF 48- AND 72-HOUR READINGS (Five Patients with Unclassifiable Reactions and Three Patients with Negative Reactions to PPD and Positive Reactions to OT Eliminated)

| Results of Readings | Number | Per Cent |
|---|---|---|
| 48- and 72-hour in agreement | 426 | 81.6 |
| 10 mm. or more at 48 hours; 5–9 mm. at 72 hours | 37 | 7.1 |
| 10 mm. or more at 48 hours; 0–4 mm. at 72 hours | 5 | 0.9 |
| 5–9 mm. at 48 hours; 0–4 mm. at 72 hours | 17 | 3.3 |
| 5–9 mm. at 48 hours; 10 mm. or more at 72 hours | 11 | 2.1 |
| 0–4 mm. at 48 hours; 5–9 mm. at 72 hours | 2 | 0.4 |
| 0–4 mm. at 48 hours; 10 mm. or more at 72 hours | 1 | 0.2 |
| Positive at 48 hours; Not read at 72 hours | 23 | 4.4 |
| Total | 522 | 100 |

TABLE 7
SUMMARY OF CHANGES FROM 48- TO 72-HOUR READINGS
(499 Classifiable Tests with Comparative Readings)

| Results of Readings | Number | Per Cent |
|---|---|---|
| Agreement | 426 | 85.4 |
| Significant decrease from 48 to 72 hours | 59 | 11.8 |
| Significant increase from 48 to 72 hours | 14 | 2.8 |
| Total significant change from 48 to 72 hours | 73 | 14.6 |
| Positive at 48 hours, Negative at 72 hours | 22 | 4.4 |
| Negative at 48 hours, Positive at 72 hours | 3 | 0.6 |

recheck testing with OT, when done, gave positive results.

In this hospital, PPD is used for routine testing, and OT only in individual instances. Therefore, no statement can be made concerning the relative efficacy of these two reagents. It would be reasonable to assume, however, that some individual differences in skin sensitivity to PPD and OT do occur, and that dubious reactions to one reagent should be checked by repeat testing with the other. If a patient shows no induration with one reagent, but definite induration with the other, the skin test should be considered to be positive. With this limitation in mind, it would be fair to state that, for all practical purposes, a negative skin reaction is reliable as an exclusion test for tuberculosis. The only exception would be in the case of the chromogenic mycobacteria in which there may be atypical skin reactions. In the single case of this type in the series presented, skin testing with tuberculin prepared from the chromogen was not done, but conceivably this could prove a useful differential diagnostic tool in such instances.

## SUMMARY

In the experience of the present writers, no cases of clinical tuberculosis have been found in which the tuberculin results were unequivocally negative at all levels of antigen and with repeated testing. Possible exceptions occur in the early infection before conversion takes place, or in the patient with overwhelming infection. Naturally, the occasional anergic patient probably exists, but no such case has been encountered in this series.

The reaction to intermediate-strength PPD is a valid screening procedure and should disclose approximately 95 per cent of reactors. However, it cannot be used as an exclusion test in the individual case. Patients showing only weakly positive reactions to second-strength PPD in the presence of clinically suspected tuberculosis were, by and large, found to be free of clinically significant tuberculous disease after thorough clinical, roentgenographic, and laboratory investigation. Patients with suspicious lesions showing no reactions to intermediate-strength PPD, but 10 mm. or more induration on testing with second-strength PPD, should be regarded as probably having positive tuberculin reactions in view of the high incidence of clinical, morphologic, and bacteriologic confirmation of active tuberculosis in this group.

A small number of patients will show negative or dubious reactions on original testing; but with repeat testing with either the same reagent, or with another tuberculin preparation, will show definitely positive results. Therefore, a negative reaction in the presence of a clinically suspicious lesion should always be confirmed.

There is a relatively small but significant variance between forty-eight- and seventy-two-hour readings so that, when possible, the reactions should be read at both forty-eight and seventy-two hours. When only one reading is feasible, the forty-eight-hour reading will give more accurate results. In any large series, there will be a small number of patients in whom no classification is possible on the basis of tuberculin testing.

## SUMARIO

*La Comprobación con Tuberculina en 530 Enfermos Recibidos en el Servicio de Tuberculosis, Hospital Militar Fitzsimons*

En su práctica, los AA. no han observado casos de tuberculosis clínica en los que los resultados de la prueba con tuberculina fueran inequívocamente negativos a todas las diluciones y con comprobaciones repetidas. Obsérvanse posibles excepciones en la infección incipiente antes de tener lugar el viraje o en el enfermo que tiene una infección agobiadora. Naturalmente, existe probablemente alguno que otro enfermo anérgico, pero

en esta serie no se ha encontrado ningún caso de tal naturaleza.

El PPD (DPP) en la concentración intermedia es un procedimiento válido de triaje y debe poner de manifiesto aproximadamente 95 por ciento de los reactores. Sin embargo, no puede usarse como prueba de exclusión en un caso dado. Los enfermos que no acusaron más que reacciones débilmente positivas a la segunda dilución del DPP cuando había clínicamente sospechas de tuberculosis, se mostraron en general exentos de afección tuberculosa clínicamente importante después de una detenida investigación clínica, radiográfica y de laboratorio. A los enfermos con lesiones sospechosas que no revelan reacciones a la concentración intermedia del DPP, pero sí induración de 10 mm. o más a la segunda dilución del DPP, debe considerárseles como teniendo probablemente reacciones positivas a la tuberculina, vista la alta incidencia de confirmación clínica, morfológica y bacteriológica de la existencia de tuberculosis activa en este grupo.

Un número pequeño de enfermos arrojará resultados negativos o dudosos en la comprobación primitiva; pero al repetirse la comprobación, ya con el mismo reactivo, o con otra preparación de tuberculina, arrojará resultados netamente positivos. Por lo tanto, hay que confirmar siempre una prueba negativa si existe una lesión clínicamente sospechosa.

Existe una variación relativamente pequeña, pero importante, entre las lecturas a las cuarenta y ocho y las setenta y dos horas, de modo que cuando es posible, deben hacerse lecturas a las cuarenta y ocho y las setenta y dos horas. Cuando no es factible más que una lectura, la de las cuarenta y ocho horas dará resultados más exactos. En toda serie numerosa, habrá un número pequeño de enfermos en los que no resulta posible la clasificación a base de la reacción a la tuberculina.


## Resume

### *Test à la tuberculine chez 530 patients admis dans le Service de la Tuberculose du Fitzsimons Army Hospital*

Selon l'expérience des auteurs, il n'a été décelé aucun cas de tuberculose clinique dans lesquels les résultats du tuberculine-test aient été incontestablement négatifs à toutes les concentrations et avec des tests répétés. Des exceptions possibles se produisent durant la période de début de l'infection avant l'apparition de l'allergie, dans certains cas d'infection foudroyante. L'existence d'un malade anergique est évidemment probable, mais ce cas n'a pas été rencontré dans cette série.

Le test avec la dilution intermédiaire de PPD est un procédé de dépistage valable; il devrait révéler approximativement 95% des sujets allergiques. Cependant il ne peut servir comme test d'exclusion dans un cas particulier. Les malades dont les réactions au PPD n'étaient que légèrement positives à la deuxième dilution lorsque la tuberculose clinique est soupçonnée ont été dans l'ensemble trouvés indemnes de tuberculose cliniquement significative après investigation comprenant des examens cliniques, radiologiques et de laboratoire. Les malades atteints de lésions suspectes ne réagissant pas au PPD en dilution intermédiaire, mais qui présentent une induration de 10 mm ou plus avec le PPD en deuxième dilution, doivent probablement être considérés comme allergiques à la tuberculine en raison de l'incidence de la confirmation clinique morphologique et bactériologique d'une tuberculose évolutive dans ce groupe.

Chez un nombre réduit de patients les résultats sont négatifs ou douteux lors du test initial, mais la répétition du test soit avec le même réactif soit avec une autre préparation de tuberculine donnera des résultats nettement positifs. En conséquence, un test négatif en présence d'une lésion suspecte cliniquement demandera toujours à être confirmé.

Il existe une variation relativement faible, mais significative, entre les lectures de la quarante-huitième et de la soixante-douzième heure de telle sorte que, quand cela est praticable, la lecture des tests sera faite à la quarante-huitième et à la soixante-douzième heure. Quand une seule lecture est possible, la lecture effectuée à la quarante-huitième heure donnera des résultats plus précis. Dans toute grande série il y aura un nombre réduit de patients chez lesquels aucune classification n'est possible sur la base du test à la tuberculine.

# Case Reports

## IDIOPATHIC HYPOVENTILATION, POLYCYTHEMIA, AND COR PULMONALE[1,2]

L. THEODORE LAWRENCE

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### Introduction

Chronic pulmonary alveolar hypoventilation is well known as a consequence of severe pulmonary disease such as obstructive emphysema. It is accompanied by anoxemia, secondary polycythemia, carbon dioxide retention, respiratory acidosis, and pulmonary heart disease. In the absence of intrinsic disease of the lungs, the same syndrome may occur as a result of constrictive pleuritis (1), extreme obesity (2, 3), the respiratory muscle weakness of poliomyelitis, or other diseases that interfere with the mechanics of respiration.

The case of hypoventilation presented here cannot be adequately explained by the preceding considerations and is similar to five other cases (4–8) which seem to be best explained by assuming a primary lack of medullary respiratory center drive. Cases 3 and 5 in the series of Newman and associates (9) may belong in this category also.

### Case Report

A young bartender was hospitalized for congestive heart failure and polycythemia three times between 1950 and 1954. Electrocardiograms showed right ventricular hypertrophy. After treatment for failure, cardiac catheterization was undertaken three times, and pulmonary arterial pressures ranged from 44/26 to 122/52. No other information of value was obtained from these procedures because of technical difficulties. Peripheral arterial blood oxygen saturation at rest was 73 per cent, rising to 98 per cent after intermittent positive pressure oxygen breathing. After apparent cardiac compensation, the blood volume was 165 ml. per kg. (marked increase above normal). Leukocyte and platelet counts were normal, and this helped to confirm the impression that polycythemia vera was not involved.

The patient was admitted to the hospital again on January 19, 1955, at the age of thirty-five. His complaints were generalized swelling and shortness of breath on walking. He had not had orthopnea or nocturnal dyspnea. He smoked about a pack of cigarettes daily. In 1943 he had had pneumonia, with amnesia for one week of the illness.

He was a short, squat man with a broad chest. His weight was 167 pounds and height, 63.5 inches. In the past he had weighed a maximum of 219.5 pounds when edematous. There was marked cyanosis. The lungs were clear except for inspiratory rales at the bases. A pulmonary systolic murmur was noted and the pulmonary second sound was loud. The liver was greatly enlarged and massive edema was present. There were no signs of phlebitis, no clubbing, and no neurologic abnormalities.

The hemoglobin concentration was 19.6 gm. per 100 ml.; the hematocrit was 66 per cent; and the leukocyte count, 6,500 per cu. mm. The urine examination was normal; the blood urea nitrogen, 15; and the carbon dioxide combining power, 28.5 mEq. per liter (35.5 on a previous admission).

On diuretics there was a rapid loss of 30 pounds, and the blood volume was then 140 ml. per kg., and the plasma volume 52 ml. per kg. by a radioactive isotope method.

Repeated chest roentgenograms showed persistent enlargement of the heart and pulmonary arteries and vascular congestion in the peripheral lung fields (figure 1).

On March 15, 1955, pulmonary function studies were performed by Dr. Hurley L. Motley. The results are summarized in table 1. It was concluded from these studies that alveolar hypoventilation was present but no emphysema, since the lung volume studies and intrapulmonary mixing were close to normal. Diffusion impairment and shunting also tended to be excluded.

A thoracotomy was performed, and no evidence of a circulatory shunt or valvular heart disease was found. A biopsy of the lingula of the left lung showed no significant emphysema, fibrosis, or vascular abnormality (figure 2).

Three neurologic consultants examined the patient and could find no specific neurologic disease. An electroencephalogram was abnormal and this was attributed to cerebral anoxia. A test for methemoglobin was negative, and on electrophoresis no abnormal hemoglobin was found.

The final admission occurred in July, 1956. Mercurial injections had kept fluid retention under fair control. For three months dyspnea had been present, and the day before admission substernal

[1] From the Medical Service of the Veterans Administration Hospital, Long Beach 4, California.

[2] This case was presented by Dr. Benjamin E. Konwaler at a Clinical-Pathological Conference at the annual meeting of the California Medical Association in Los Angeles, April 29, 1958.

FIG. 1. Roentgenogram of April 1, 1955, showing an unusually short, broad chest with enlargement of the heart and pulmonary arteries.

pain and hoarseness appeared. On examination of the chest, there were fine crackling rales up to the scapulae. In addition to previous cardiac findings, a pericardial friction rub was noted. The blood pressure was 110 systolic and 80 diastolic in mm. of mercury. Cyanosis and jaundice were present. The liver was enlarged and the legs were edematous. Polycythemia was noted as before, and the blood urea nitrogen was elevated. In spite of phlebotomy, artificial respiration, adrenal steroids, and other measures, death occurred in a few days.

*Autopsy findings:* At autopsy the anteroposterior diameter of the chest appeared normal. There was dependent edema and a small amount of fluid in the pleural and peritoneal cavities. Subcutaneous and abdominal fat deposits were normal in amount but pinker than usual because of vascular engorgement which was noticeable also in most of the visceral organs.

The left lung weighed 600 gm. and the right, 800 gm. There were moderate black anthracotic markings. The pleural surfaces were normal as were the bronchial structures, except that they contained considerable pink, frothy material. Scattered throughout the lungs, particularly at the bases, were many regions of consolidation, 1.5 to 2.0 cm. in diameter. On section they appeared to be infarcts; proximal to these were friable, adherent clots in the small arteries. The remainder of the lungs showed edema. The pulmonary arteries were larger than normal and contained marked atherosclerosis.

The heart weighed 600 gm., and both ventricles were hypertrophied, the right ventricular wall being 1.2 cm. in thickness and the left 1.5 cm. The myocardium was reddish-brown with some streaks of yellowish discoloration on the endocardial surface. There were no mural thrombi. The papillary muscles were hypertrophied. Atheromatous plaques were present on the aortic and pulmonary valve leaflets, but there was no evidence of stenosis or insufficiency.

The coronary arteries were patent but showed patchy areas of narrowing due to atheromatous plaques. There were fibrinous deposits on small areas of the epicardium and moderate arteriosclerosis of the aorta.

The abdominal organs were congested. The spleen weighed 150 gm. The abdominal and periprostatic veins were free of thrombi.

The nervous system was examined with particular care. The brain weighed 1,550 gm. The meninges were normal. The basilar artery and its branches were all patent and showed no evidence of thrombosis. All of the cerebral vessels were

engorged, but the brain was not edematous. Many fine sections taken through the pons and brainstem were normal.

Microscopic sections of the lungs showed areas of infarction. A moderate number of pigment-containing histiocytes were seen in the alveolar spaces. The small pulmonary arteries showed subintimal and medial thickening. There were many thrombi, some fresh and some undergoing various degrees of organization. Minimal pulmonary emphysema and fibrosis were present (figure 3).

The myocardium was normal. The pericardium showed minimal round-cell infiltration. The brainstem sections were morphologically normal. The spleen was normal except for congestion. The liver showed central congestion and necrosis. The bone marrow was hypercellular. Sections of phrenic nerve and skeletal muscle were normal, and there were no important findings in the other organs.

The pathologist thought that the pulmonary infarcts were rather recent in histologic appearance and could not be considered a primary factor in the illness. They were probably caused by stasis due to heart failure and polycythemia.

## Discussion

Peripheral arterial anoxemia may be caused by a circulatory shunt, impaired diffusion of oxygen from the alveoli to the blood, or inadequate ventilation of the alveoli. The last occurs in emphysema due to airway obstruction and impaired mixing of inspired air and may also result from deficient bellows action of the chest. This report is concerned with deficient bellows action related to lack of respiratory center drive.

The peculiar phenomena observed led to a search for evidence of polycythemia vera, congenital or valvular heart disease, and emphysema. None of these could be diagnosed.

Analysis showed the following important features: Chronic hypoventilation which could be corrected voluntarily; arterial oxygen unsaturation and polycythemia; pulmonary hypertension and right heart failure; left ventricular hypertrophy; autopsy finding of pulmonary arteriolar thrombosis of relatively recent origin; absence of morphologic changes in the central nervous system.

Some of the possible relationships of these factors are indicated in figure 4.

Early in the course of observation of this patient it became clear that arterial oxygen unsaturation was the cause of his polycythemia, pulmonary hypertension, and congestive failure. The pulmonary function studies of Dr. Motley

TABLE 1

Summary of Pulmonary Function Studies of March 15, 1955

Volume Studies

Vital capacity, supine—3,274 cc. (95% of predicted)
Vital capacity, standing—3,612 cc. (105% of predicted)
Three-second vital capacity, standing—3,612 cc. (normal)
Maximal breathing capacity—143 L/min. (108% of predicted); no significant change after isoproterenol
Alveolar nitrogen after breathing oxygen for 7 minutes—1.32% (normal intrapulmonary gas mixing)
Residual air—894 cc. (78% of normal, moderate decrease)
Total lung capacity—4,168 cc. (91% of normal, slight decrease)
Residual air—21.4% of total lung capacity (normal)

Studies During Room Air Breathing without Mouthpiece and Nose Clip

Average tidal air at rest—325 cc. (low)
Calculated physiologic dead space (based on expired $p_{CO_2}$ and arterial $p_{CO_2}$)—101 cc.
Effective tidal air—69% (normal 75%)
Arterial oxygen saturation—74.2%; pH—7.36
Arterial oxygen saturation after voluntary deep, fast respiration for a few minutes—97%; pH—7.48

Studies on 100% Oxygen Breathing

Arterial oxygen saturation after 100% oxygen, 6 minutes—98.5%; $CO_2$ content—70 vol. per cent; pH—7.38

Studies during Compressed Air Breathing Using Mouthpiece and Nose Clip

Arterial oxygen saturation at rest—86.7%; pH—7.37
Arterial oxygen saturation during step-up exercise—76%
Arterial $CO_2$ at rest—64 vol. per cent (increased)
Arterial $CO_2$ after step-up exercise—60 vol. per cent
Arterial oxygen saturation at rest with intermittent positive pressure breathing—96%; $CO_2$—48 vol. per cent (normal); pH—7.59 (hyperventilation)
Arterial blood at rest: $p_{CO_2}$—67.4 mm. Hg (normal 40); $p_{O_2}$—65.1 mm. Hg (normal 95)

Pulmonary Ventilation Measurements at Rest

Respirations per minute—17 (normal 14 to 22)
Ventilation, L/min./sq. M. BSA—3.29 (normal 2.6 to 3.8)
Oxygen uptake, ml./min./sq. M. BSA—176 (normal 120–145)
Per cent oxygen extracted from inspired air—5.37 (normal 4 to 5)

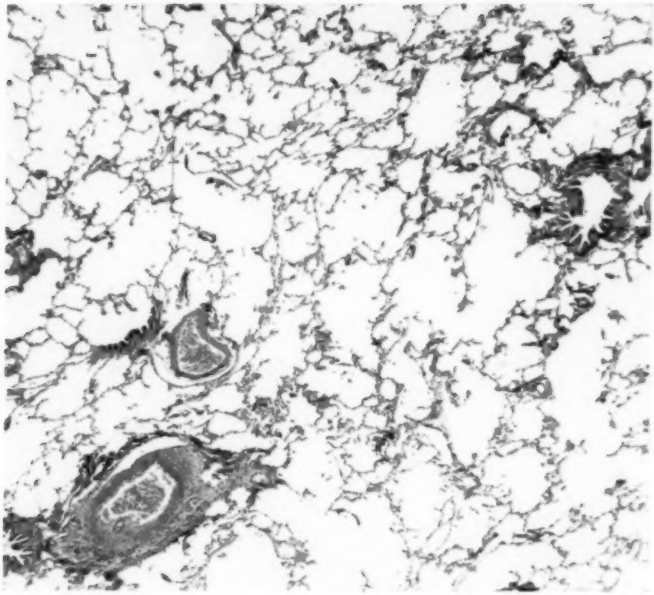

FIG. 2. Biopsy of the pulmonary lingula, April 19, 1955, showing evidence of some surgical trauma but an absence of diffuse inflammation or fibrosis. The fine pattern of alveolar septa indicates essentially normal structure. (× 35)

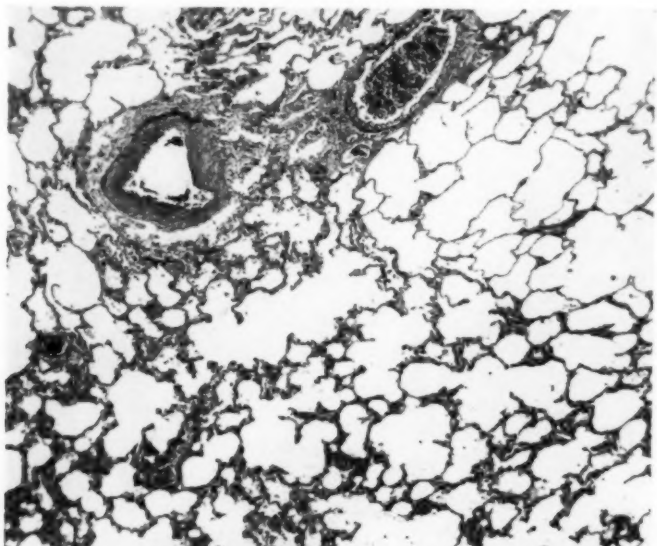

FIG. 3. Autopsy specimen of lung showing an area away from the infarcts. Arterial thickening, congestion, and an absence of interstitial fibrosis are present. Some of the abnormalities may be related to infarction in adjacent lung tissue. (× 35)

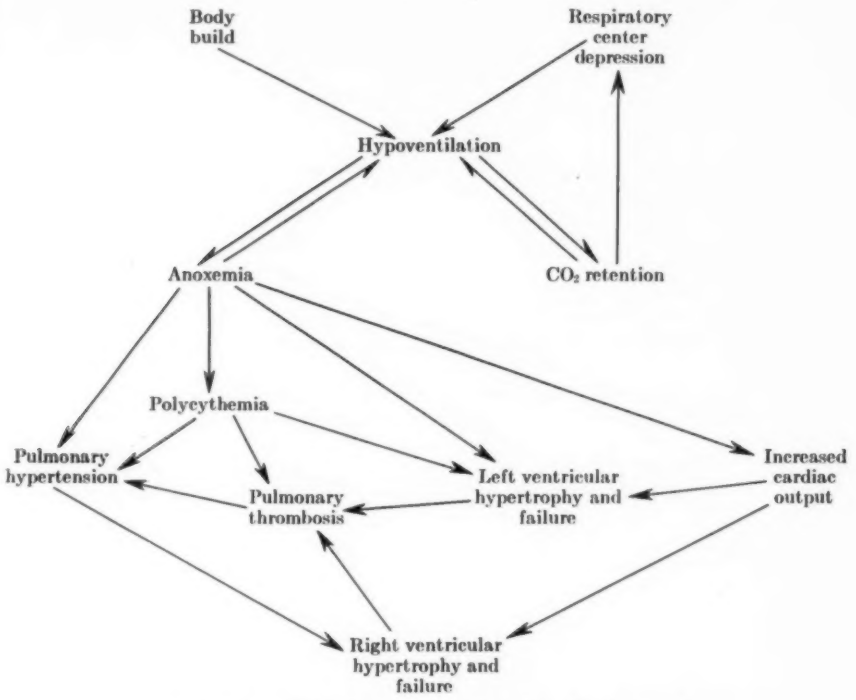


FIG. 4. Diagram showing partial explanation of pathogenesis.

(table 1) were crucial in determining the cause of the anoxemia.

The first possibility considered was obstructive emphysema. This was rejected because none of the characteristics of emphysema were found in the lung volume studies. The total lung capacity and residual air were low rather than high, as in emphysema. The vital capacity and three-second vital capacity were both normal, indicating no airway obstruction; and alveolar nitrogen was rapidly eliminated on oxygen breathing, showing efficient intrapulmonary gas mixing.

A second possibility was an alveolar capillary diffusion block, as in the Hamman-Rich syndrome and various other pulmonary interstitial diseases. This defect causes arterial oxygen unsaturation which is readily overcome by breathing an increased oxygen concentration, but it is not apt to cause carbon dioxide retention since carbon dioxide diffuses about twenty times as easily as oxygen. The increased arterial carbon dioxide tension, therefore, tended to eliminate this possibility.

Right to left shunting is a third cause of severe anoxemia; but this cannot be corrected by oxygen breathing or hyperventilation, nor is it likely to be associated with carbon dioxide retention.

Finally, even in the absence of emphysema, alveolar hypoventilation may cause anoxemia and in this condition carbon dioxide retention is a cardinal sign. Alveolar hypoventilation frequently occurs acutely as a result of morphine and anesthesia. The best known chronic example is perhaps the obesity hypoventilation syndrome. It has been suggested that the "friction" involved in moving the excess fat in the chest wall and abdomen encourages shallow, inadequate respiration. Voluntary hyperventilation usually corrects the deficiency, and this was true also in the present case. Another similarity was the moderate decrease in lung volume and residual air which is found in the obesity hypoventilation syndrome. The patient may have presented an example of this syndrome although most observers were impressed that he had a short, stocky build rather than that he was fat. Even-

tually he lost a large amount of weight but this did not prevent progression of the disease, as it has in some reported cases of the obesity syndrome. Furthermore, obesity alone is not necessarily a satisfactory explanation of hypoventilation. This has been shown by Bedell and associates (10) who found some very obese persons without anoxemia and others with anoxemia which could be explained by conditions other than the obesity.

In considering hypoventilation of any origin it should be remembered that carbon dioxide retention normally acts as a potent stimulus to the respiratory center so that the excess carbon dioxide is then eliminated. If this response does not occur, carbon dioxide subsequently depresses the center. Anoxemia also has a depressing effect but this is normally more than overcome by the reflex stimulation caused by anoxemia via the aortic and carotid nerves.

Brown (11) has reported three "normal" medical students who failed to show increased ventilation on breathing 8 per cent oxygen. Perhaps, in time, such persons would develop chronic hypoventilation since anoxemia becomes an essential stimulus to respiration under certain circumstances such as high altitude.

Acute bulbar poliomyelitis often causes erratic function of the medullary respiratory center with hypoventilation, and this defect may persist in some cases (12). It is usually associated with other bulbar signs such as cranial nerve palsies. The patient of the present case had a history of severe "pneumonia," and this suggests that an infectious disease may have damaged his respiratory center.

Pulmonary vascular occlusion, anoxia, and polycythemia may each contribute to pulmonary hypertension. The patient did not seem to have chronic pulmonary embolism or thrombosis because he differed from the patients in the reported cases (13) in having a greater degree of polycythemia, in the absence of hemoptysis, in having minimal dyspnea, and increased rather than decreased peripheral pulmonary vascular markings on the roentgenogram. The pulmonary arteriolar thrombosis was presumably only terminal.

Roentgenograms in this case were repeatedly interpreted as showing peripheral pulmonary vascular engorgement. This might be attributed to left heart failure but seems more likely to have been caused by the greatly increased blood volume. This phenomenon is recognized in polycythemia vera. It has been shown that the blood volume is usually increased in secondary as well as in primary polycythemia (14). This is not apparent in the lungs in emphysema because the pulmonary vascular bed is reduced, or in cyanotic congenital heart disease in which pulmonary circulation is reduced; but may be seen in the polycythemia of hypoventilation states when the lungs are essentially normal.


## Summary

A 37-year-old man died after a six-year illness based on pulmonary alveolar hypoventilation which caused polycythemia and pulmonary heart disease. Defects of any part of the respiratory apparatus may cause hypoventilation. This case is unusual in that the main defect seemed to be a functional one of the medullary respiratory center. Several cases of this type have been reported, but this is believed to be the only one examined at autopsy and the absence of significant findings in the brain stem is of interest.

## Sumario

*Hipoventilación Idiopática, Policitemia y Cor Pulmonar*

Un sujeto de 37 años falleció después de una enfermedad de seis años, basada en una hipoventilación alvéolopulmonar que ocasionó policitemia y cardiopatía pulmonar. Los defectos de cualquier parte del aparato respiratorio pueden ocasionar hipoventilación. Este caso es extraño por parecer el defecto principal ser funcional, del centro respiratorio en la médula oblongada. Se han descrito varios casos de este género, pero este enfermo parece ser el único examinado en la autopsia, y reviste interés la falta de hallazgos importantes en la médula oblongada y el puente de Varolio.

## Resume

*Hypoventilation idiopathique, polycytémie et coeur pulmonaire*

Une homme âgé de 37 ans est mort au bout de six ans d'une maladie dépendant d'une hypoventilation des alvéoles pulmonaires qui entraînait une polycytémie et une maladie cardio-pulmonaire. L'altération de toute partie du système respiratoire peut engendrer l'hypoventilation. Le cas cité est inhabituel en ce sens que l'altération principale consistait en un trouble fonctionnel du centre respiratoire médullaire. Plusieurs cas de ce

type ont été rapportés, mais on croit que celui-ci est le seul qui ait été examiné à l'autopsie; l'absence de signes significatifs dans le tronc cérébral est remarquable.

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# *Editorial*

## Air Pollution and Bronchitis[1]

"Chronic bronchitis," which in our medical school days was already an obsolescent diagnosis, is appearing with increasing frequency in the medical publications of recent years, due mainly to the great importance attached to it in British literature. It is only fair to say, in behalf of our British colleagues, that in reviving interest in such a weather-beaten "catch-all" they are not moved by choice, but rather by the impact of a very serious situation that has created in their land a complicated syndrome answering to that name.[2, 3]

British "chronic bronchitis" does not refer to the benign, inconsequential "bronchial trouble" the name is apt to evoke in our minds. It applies instead to a progressive respiratory disorder in which the factor of bronchial disturbance is a constant denominator. As such, the name possesses undisputed validity.

The typical sequence of events observed in this condition can be outlined as follows: Its onset may be either insidious (52 per cent) or acute (48 per cent). In cases of insidious onset, the picture may be at first indistinguishable from what we would refer to as a "chest cold" or a "smokers' bronchitis"—as much a run-of-the-mill occurrence there as it is here. Some of these cases, however, go on to a more severe form of recurrent bronchitis with variable degrees of disability due to cough and sputum during the winter months. Still later on, the bronchitis becomes a year-round affair with intercurrent exacerbations. When the onset of the disease is acute, the intermittent stages may be dispensed with and respiratory invalidism sets in at an early date. Shortness of breath due to bronchial spasm may ensue at any time during the initial stages, particularly in association with acute flare-ups.[2, 4]

As the bronchitic process advances, the lung parenchyma is involved and emphysema gradually develops to aggravate the situation. The dividing line between the degree of respiratory embarrassment that is due to bronchial spasm or to the anatomic disruption of emphysema is poorly defined due to the overlapping of both factors and their frequent coexistence. In fact, emphysema is such a constant feature of this disease that, according to Oswald, "from a clinical standpoint, the question is not so much whether the chronic bronchitic has emphysema or not, but how much emphysema he has." These bronchitics are notoriously prone to frequent bouts of bronchopulmonary superinfection which further deteriorate their dwindling respiratory reserve and render them hopeless cripples. Cor pulmonale, as could be anticipated, is often an associated complication.

Up to this point, we must admit, the situation described would seem to differ little from what our own clinicians observe in cases of chronic nonspecific bronchopulmonary disease. The importance of the chronic bronchitis syndrome in England and Wales lies in its socio-economic aspects.

Among a total population of some 45 million, chronic bronchitis outranks all other respiratory diseases as a crippler and a killer. It accounts for an annual loss of nearly 27 million working-man days, and it is given as the cause of death in 30,000 certificates every year. It is responsible for almost twice as many deaths as respiratory cancer (16,000 for 1953) and easily triples the figures for tuberculosis in this respect (8,000 for 1953).[2]

On account of these overwhelming figures due to a disease with such unspecific features, the factors involved in the etiology of chronic bronchitis have been the subject of intense scrutiny for many years. The role of infections, environmental, genetic, and even endocrine influences have all been emphasized with more or less plausible evidence. Although all or any of those are likely to play a part, there still remain some disconcerting elements not entirely understood.

Probably the most important single element in chronic bronchitis is infection, both as a causative and an aggravating agent. In established cases of bronchitis the most commonly found organisms are *Hemophilus influenzae* and pneumococci, plus the common bacterial flora of respiratory infections. *H. influenzae* is considered the most important.[2, 5, 6] These organisms are likely to be

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found in cases of bronchitis when the sputum is purulent. They are also found, however, in subjects with mucoid sputum.

The persistent sputum of bronchitics is baffling, and the effects of antimicrobial therapy upon the quality and quantity of that sputum present some unusual angles. When sputum is purulent, antimicrobial therapy often succeeds in reducing the amount and in converting the secretions to a mucoid character. Yet, when the patient has only mucoid sputum to start with, chemotherapy fails entirely to reduce its amount or to sterilize it. Furthermore, clinical improvement is rarely achieved by the use of antimicrobial drugs. In some cases the reduction of purulent expectoration to a scant mucoid discharge might even worsen a patient's condition, probably due to the increased viscidity of his sputum.[7, 8] These paradoxical facts have promoted great interest in the possible viral nature of the irreducible bronchorrhea.[9]

The likelihood of an environmental causation of chronic bronchitis has long been entertained. Following the smog episodes of 1948, 1952, and more recently 1956 and 1957,[10-14] when bronchitics paid a heavy toll, the search for corroborating evidence in this direction gained much impetus. Some of the studies on this subject are very illuminating.

When Pemberton and Goldberg (University of Sheffield) analyzed the bronchitis mortality rates for England and Wales in 1951, they discovered a striking relationship between these rates and the size of the communities in which they occurred. Their findings also illustrated that sex differences are quite consistent in all groups.[15]

Further analysis of their data revealed a highly significant correlation between bronchitis mortality and the average sulfur-dioxide concentration in the different areas—a correlation that makes lots of sense when it is recalled that sulfur dioxide is the most suspected atmospheric pollutant in terms of health effects. Interestingly enough, pollution by other materials like solid matter, which settles over England and Wales at the rate of some 2.5 million tons per year, did not agree too well with the mortality rates. Nor did other important parameters like social conditions.

Fairbairn and Reid (London School of Hygiene) contrasted morbidity and mortality trends in the middle-age groups with environmental conditions in the United Kingdom and found that the figures for bronchitis related keenly to a simple air pollution index based on atmospheric visibility.[16]

Reid (1956) compared invaliding rates from bronchitis in London postmen with the varying concentrations of smoke pollution over that city as determined by the direction of prevailing winds and found also a remarkable correspondence.[17]

Lawther[2, 13, 18, 19] has been following the day-to-day variations in the condition of bronchitics in London for the last several years and comparing their changes to smoke and sulfur-dioxide concentration in the London air. His data clearly show that bronchitis is definitely aggravated by atmospheric pollution, particularly during the winter months when pollution is highest.

An affair of such magnitude as chronic bronchitis is in England cannot fail to awaken our interest as medical men. In view of the apparent relationship of that disease to atmospheric pollution—a problem that is of increasing importance to us, both as physicians and private citizens—that interest becomes far more than academic. There would seem to be little reason for emphasizing the necessity of clearly identifying an American counterpart to the British syndrome which we could keep under close surveillance to assess air pollution influences on human health. Integrating such counterpart by pooling data on a variety of respiratory disorders seen in America that are identifiable with the British syndrome is, unfortunately, not feasible at this moment. We shall wait until completion of a number of surveys now in progress to undertake a meaningful comparison of American and British statistics in the months ahead.

If we compare current mortality figures for chronic bronchitis in America with those of England and Wales, the differences are too enormous to be taken seriously as representative,

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[17] Reid, D. D.: Proc. Roy. Soc. Med., 1956, *49*, 767.

[18] Lawther, P. J.: Proc. Roy. Soc. Med., 1958, *51*, 262.

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British figures being approximately thirty times greater than ours![20, 21]

If we resort to figures on morbidity, our chances are perhaps better for the simple reason that the average American physician is far less reluctant to attach a diagnosis of chronic bronchitis to a common respiratory illness than to a major condition capable of causing death. Still, when we plotted the estimated prevalence of bronchitis in the adult male population of New York City against Reid's data for male workers at the London Post Office, our rates were something like three to four times lower in the older age groups.

While the reasons responsible for those differences could be speculated upon forever, it may be well to point out that, when specifically looked for, chronic bronchitis is detected in this country with greater frequency. Phillips and associates, at the Veterans Hospital, Providence, Rhode Island, investigating the main cause of chronic cough in adult males, found that, in the majority of the cases, such chronic cough was not due to tumors, tuberculosis, asthma, bronchiectasis, pneumonitis, or what-not, but rather to old-fashioned chronic bronchitis, which they related to the irritating effects of cigarette smoking—a particular variety of air pollution, if you please. Moreover, the description of the course of illness in their patients closely resembled the sequence observed in English bronchitis. In 20 cases of emphysema examined at autopsy, they also reported a one hundred per cent incidence of chronic bronchitis.[22] Had it not been for the particular interest of those investigators in this subject, I have serious doubt that any mention of chronic bronchitis would have ever been entered in the histories and death certificates of those patients. It may be said, parenthetically, that the accuracy of our figures on emphysema does not seem to fare much better. One has only to think of the frequency with which advanced emphysema is encountered clinically and the relatively low death rates recorded for that condition.

Gaensler,[23] impressed by British reports on the association of chronic bronchitis and emphysema, made a retrospective study of 100 consecutive cases of pulmonary emphysema, physiologically diagnosed in his laboratory at the Boston City Hospital. When he applied the British diagnostic criteria of chronic bronchitis to the case histories, he was surprised to find an incidence of 68 per cent.

Probably the one conclusion that can be drawn from all of this is that the incidence of chronic bronchitis is grossly underestimated in our country and that perhaps the disease is overdiagnosed in Britain to some extent.[24, 25] When data now being obtained by improved survey methods in both countries become available, we may be able to establish a valid estimate on the importance of chronic bronchitis among us. Armed with such knowledge, we shall then seek to clarify the relationship of that disease to air pollution in our cities.

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# *Notes*

## A SIMPLE PAPER STRIP URINE TEST FOR PARA-AMINOSALICYLIC ACID

Several studies have demonstrated the usefulness of a urine test for para-aminosalicylic acid (PAS) or its metabolites in ambulatory tuberculous patients.[1-4] However, the simplest of these tests, the one using ferric chloride solution, has proved to be too cumbersome for routine use in the chest clinics of the Bureau of Tuberculosis. The writer was most receptive, therefore, to a suggestion made by a nursing consultant[5] that a new dip-and-read ferric ion paper strip test,[6] now commercially available as a simplified substitute for the ferric chloride test for phenylketonuria,[7-9] might similarly be found to be a practical field test for PAS and its metabolites in the urine. This possibility was investigated with the assistance of a group of cooperating physicians in seven different hospitals in New York City.

The tests consisted of dipping into the urine a stiff strip of cellulose impregnated with ferric and magnesium ions plus cyclohexyl sulfamic acid[10] and observing the color at the test end of the strip thirty seconds after taking it out of the urine. PAS or its urinary metabolites were found to impart a color ranging from light pink, lavender, or violet to dark purple. Any color change in this range was considered to be a "positive" test. The urine of patients receiving other derivatives of salicylic acid in the usual doses frequently caused the test end of the strip to turn light pink to violet, but this was not confusing if a careful history of drug ingestion was taken. All other color changes were considered as "negative" for PAS. Details of the significance of all of the various colors that might be imparted by a variety of urinary metabolites to the strip reagent are discussed elsewhere.[7-9] The presence of glycosuria did not affect the test. Quantitative studies to correlate the concentration of PAS or its metabolites in the urine with a scale of color changes have not been done to date.

TABLE 1

RESULTS OF PAPER STRIP (PHENISTIX®) URINE TEST FOR PARA-AMINOSALICYLIC ACID IN 256 HOSPITALIZED PATIENTS

| Patient Group | Total Tests Done | Positive* | Negative† |
|---|---|---|---|
| PAS prescribed | 149 | 145 | 4 |
| Regular dose taken‡ | 145 | 145 | 0 |
| 1 gm. three times daily§ | 1 | 0 | 1 |
| PAS not taken‖ | 3 | 0 | 3 |
| PAS not prescribed | 107 | 14 | 93 |
| Other salicylates prescribed | 21 | 12 | 9 |
| No salicylates prescribed | 86 | 2¶ | 84 |

* Any color ranging from light pink, violet, or lavender to dark purple.

† Any other color.

‡ 3 to 4 gm.; urine collected 1 to 12 hours after test dose.

§ Urine collected 12 hours after test dose.

‖ Discovered only after result of test became known.

¶ One patient was taking acetylsalicylic acid without a physician's order; the other patient left the hospital before further investigations could be carried out.

A single urine test was done on 149 hospitalized patients for whom PAS was known to be prescribed (table 1). Urine specimens were collected from one to twelve hours after the test dose (3 or 4 gm.) was administered. In addition, urine was collected from 107 patients who were not receiving PAS. All of the 256 urine specimens were tested without knowledge of the type of medication the patients represented were receiving.

Of the 149 "PAS" patients, 145 had urine which

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[3] FOX, W.: Tubercle, 1958, *39*, 269.

[4] BREITE, M. J.: Am. Rev. Tuberc., 1959, *79*, 672.

[5] Mildred C. Lant, Bureau of Nursing, New York City Department of Health.

[6] Phenistix, manufactured by Ames Company, Inc., Elkhart, Indiana.

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[9] WORTIS, J., AND GIANCOTTI, A. M.: Am. J. Pub. Health, 1959, *49*, 463.

[10] Supplied by Dr. N. L. Heminway, Medical Director, Ames Company, Inc.

was "positive," most specimens imparting a dark purple reaction to the paper strip reagent. Further investigations of the 4 patients with "negative" urine tests revealed that one was taking 1 gm. of PAS three times daily, having taken the last dose twelve hours prior to urine collection, while the remaining 3 patients had been surreptitiously discarding all of their PAS medication for several days.

Of the 107 patients not receiving PAS, 21 were known to be taking some other form of salicylates (usually acetylsalicylic acid) at the time of the urine collection. In 12 of these patients, the urine test was weakly "positive"; in 9 the test was "negative." In the remaining 86 patients, 84 had "negative" tests and 2 had weakly "positive" reactions. One of the latter patients was found to be taking acetylsalicylic acid without a physician's order, and detailed questioning of the other patient could not be carried out because of an irregular discharge from the hospital shortly after the urine was collected.

One of the physicians[11] cooperating in this study took 5 gm. of PAS and then tested her urine specimens with both the ferric ion paper strip and 3 per cent ferric chloride solution at three-, five-, nine-, eleven-, thirteen-, sixteen-, and twenty-one-hour intervals after ingestion. Both methods gave "positive" tests at all intervals up to sixteen hours after ingestion. At twenty-one hours, however, the paper strip test was "negative," whereas the ferric chloride solution test was still faintly "positive."

* * *

It may be inferred from the present findings that the ferric ion paper strip test is an effective one for detecting PAS in the urine collected from patients within twelve hours of taking 3 gm. or more of this drug. The test seems to be almost as sensitive as the 3 per cent ferric chloride method, and is certainly much easier to perform and interpret. On the basis of these studies, the paper strip test for PAS has now been incorporated into the regular routine of supervision of ambulatory patients for whom PAS has been prescribed in all 25 chest clinics of the Bureau of Tuberculosis. Thus far, the test has proved to be practical for field use even in the busiest clinics, and it has already provided the clinic personnel with a good deal of useful information. It is to be hoped that more details of the sensitivity and specificity of this test will soon be forthcoming.

A similar type of test for the detection of isoniazid in the urine to replace the complicated tests presently employed[12] is also urgently needed to provide another important guide for the supervision of patients receiving long-term antituberculous chemotherapy.

*In summary:* A dip-and-read ferric ion paper strip test (Phenistix®) was found to be a readily performed and an easily interpreted method of detecting PAS or its metabolites in the urine of patients receiving the usual dose of this drug. This simple test has already proved to be a practical and useful tool in the supervision of ambulatory tuberculous patients attending busy chest clinics.

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[11] Dr. E. S. Nash, Bellevue Hospital, New York, New York.

[12] GANGADHARAM, P. R. J., *et al.*: Tubercle, 1958, *39*, 191.

# THE EFFECT OF TOPICAL HYDROCORTISONE ACETATE OINTMENT ON THE TUBERCULIN SKIN REACTION

Preliminary observations on a small number of hospitalized tuberculous patients[1] suggested that the application of hydrocortisone acetate ointment at the site of tuberculin injection twenty-four hours after the performance of the test might be useful in preventing unnecessarily severe reactions and in minimizing undesirable side effects of tuberculin hypersensitivity.

This method was used in a group of 142 patients hospitalized at National Jewish Hospital and Fitzsimons Army Hospital at Denver. The purpose of this paper is to report the results of this study.

The tuberculin used in this study was commercial purified protein derivative of tuberculin (PPD), intermediate strength (5 TU). The hydrocortisone ointment used consisted of 1 per cent hydrocortisone acetate, U.S.P., with 0.025 per cent benzalkonium chloride in a petrolatum base. In order to equalize external conditions, rose-water ointment, U.S.P., was applied at the control site of the tuberculin injection.[2]

The tuberculin skin reactions were classified according to *Diagnostic Standards* of the National Tuberculosis Association (1955) as: negative, when no induration was palpable at the injection site; slight (+), 6–10 mm. of induration; moderate (++), 11–20 mm. of induration; marked (+++), more than 20 mm. of induration; and, severe (++++), when central necrosis accompanied the reaction. Differences between the control and treated reactions of each individual were compared on the basis of these categories.

Intermediate strength PPD, 0.1 ml., was injected by the intracutaneous route into each forearm of a group of 142 patients. Twenty-four hours after the injection, the induration on both forearms was measured. Only those patients who had identical reactions at both sites were included in this study. Thus, the total number of patients was reduced to 110. Since precise quantitation of the response to hydrocortisone acetate ointment could not be made in the remaining 32 patients, it was considered proper to eliminate them from further consideration.

A circular surface of skin, 1 inch or more in diameter, at one of the injection sites (called the treated site) was covered with a thin layer of

TABLE 1

EFFECT OF TOPICAL HYDROCORTISONE ACETATE OINTMENT ON THE PROGRESS AND ULTIMATE FATE OF INTRACUTANEOUS TUBERCULIN REACTIONS

| Number of Cases | Type of Reaction 24 Hours after Test* | Type of Reaction 48 Hours after Test | | | | | | | | | | Type of Reaction 72 Hours after Test | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Negative | | Slight | | Moderate | | Marked | | Severe | | Negative | | Slight | | Moderate | | Marked | | Severe | |
| | | C† | T‡ | C | T | C | T | C | T | C | T | C | T | C | T | C | T | C | T | C | T |
| 14 | Negative | 11 | 11 | 2 | 2 | 1 | 1 | — | — | — | — | 10 | 10 | 3 | 2 | 1 | 2 | — | — | — | — |
| 16 | Slight | — | — | 5 | 10 | 10 | 6 | — | — | 1 | — | 4 | 3 | 1 | 5 | 9 | 8 | — | — | 2 | — |
| 66 | Moderate | 1 | 1 | 8 | 12 | 53 | 49 | 4 | 4 | — | — | 4 | 3 | 14 | 11 | 42 | 47 | 1 | 2 | 4§ | 2§ |
| 14 | Marked | — | — | 1 | 2 | 6 | 7 | 3 | 3 | 4 | 2 | — | — | 1 | 1 | 5 | 8 | 2 | 2 | 5§ | 2§ |
| 0 | Severe | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| 110 | Totals | 12 | 12 | 16 | 26 | 70 | 63 | 7 | 7 | 5 | 2 | 18 | 16 | 19 | 19 | 57 | 65 | 3 | 4 | 11 | 4 |

* Each patient had identical tuberculin reaction on both left and right forearms at 24 hours after injection of the tuberculin.

† Control site of intracutaneous tuberculin injection.

‡ Topical hydrocortisone acetate ointment to tuberculin reaction site at 24 hours and again at 48 hours after the injection of tuberculin.

§ One reaction was not measured.

[1] LEVY, D., AND RUSSELL, W. F., JR., Am. Rev. Tuberc., 1959, *79*, 666.

[2] The petrolatum base with 0.025 per cent benzalkonium chloride was shown by control, double-blind, testing on seven adult tuberculous patients to cause no change in the size or form of the tuberculin reaction or to alter the side effects of tuberculin hypersensitivity. The rose-water ointment used was also shown to cause no change in the size or form of the reactions of an additional group of 7 patients, nor to alleviate the itching and pain at the site of the injections.

hydrocortisone acetate ointment. The injection site on the right forearms of 42 patients was treated with hydrocortisone ointment. The site on the left forearms of the remaining 68 patients was treated with hydrocortisone ointment. The opposite site (called the control site), was covered with a similar layer of rose-water ointment. Both sites were massaged gently. Forty-eight hours after the injection, hydrocortisone ointment and rose-water ointment were applied once more at the respective sites and massage was carried out as above.

The patients did not know what ointments were used or what reactions to expect. They were instructed to record and report any feeling or sensation at the injection sites.

The extent of induration at the injection sites was measured at twenty-four, forty-eight, and seventy-two hours after the injection. At National Jewish Hospital the measurements were performed by a physician who did not know whether a treated or a control reaction was being read. At Fitzsimons Army Hospital the induration size was measured independently by two physicians; when a difference in the measurement existed, the higher figure was recorded.

The side effects of tuberculin hypersensitivity (itching, soreness, and pain) were recorded as present or absent.

The following criteria were adopted for interpretation of the results of this experiment: total inhibition of reaction, when there was complete absence of induration as compared with a positive reaction at the control site; partial inhibition, when the induration was less pronounced than that of the control reaction; absence of inhibition, when there was no difference; and, aggravated, when the treated site showed a more pronounced reaction than the control site.

The development and ultimate fate of each reaction related to the mode of management are shown in table 1. At twenty-four hours after the injection, 14 patients had negative reactions on both forearms; 16 patients had slight reactions; 66 had moderate reactions; and 14 had marked reactions. There were no severe reactions at twenty-four hours.

The results of the investigation are summarized in tables 1 to 4. The comparison of the reactions at the control site with the counterpart treated in-

TABLE 2

INFLUENCE OF TOPICAL HYDROCORTISONE ACETATE OINTMENT AT SITE OF INTRACUTANEOUS TUBERCULIN INJECTION ON THE SIZE OF THE 48-HOUR INDURATION

| Type of Control Reaction | Number of Cases | Type of Reaction at Site Treated with Hydrocortisone Acetate Ointment | | | | |
|---|---|---|---|---|---|---|
| | | Negative | Slight | Moderate | Marked | Severe |
| Negative | 12 | 12 | — | — | — | — |
| Slight | 16 | — | 13 | 3 | — | — |
| Moderate | 70 | — | 12 | 56 | 2 | — |
| Marked | 7 | — | — | 3 | 4 | — |
| Severe | 5 | — | 1 | 1 | 1 | 2 |
| Totals | 110 | 12 | 26 | 63 | 7 | 2 |

TABLE 3

INFLUENCE OF TOPICAL HYDROCORTISONE ACETATE OINTMENT AT SITE OF INTRACUTANEOUS TUBERCULIN INJECTION ON THE SIZE OF THE 72-HOUR INDURATION

| Type of Control Reaction | Number of Cases | Type of Reaction at Site Treated with Hydrocotisone Acetate Ointment | | | | |
|---|---|---|---|---|---|---|
| | | Negative | Slight | Moderate | Marked | Severe |
| Negative | 18 | 14 | 3 | 1 | — | — |
| Slight | 19 | — | 6 | 13 | — | — |
| Moderate | 57 | 2 | 7 | 47 | 1 | — |
| Marked | 3 | — | 1 | — | 2 | — |
| Severe | 11 | — | 2 | 4 | 1 | 4 |
| Totals | 108 | 16 | 19 | 65 | 4 | 4 |

TABLE 4

INFLUENCE OF TOPICAL HYDROCORTISONE ACETATE OINTMENT AT SITE OF TUBERCULIN INJECTION ON THE APPEARANCE OF SIDE EFFECTS OF TUBERCULIN HYPERSENSITIVITY

| Type of Reaction | Site | Presence of Side Effects of Tuberculin Hypersensitivity | | | | | |
|---|---|---|---|---|---|---|---|
| | | 48 Hours after Test | | | 72 Hours after Test | | |
| | | Number of Cases | Cases with Side Effects | | Number of Cases | Cases with Side Effects | |
| | | | Number | Per Cent | | Number | Per Cent |
| Negative | Control | 12 | — | — | 18 | 1 | 5 |
| | Treated | 12 | — | — | 16 | — | — |
| Slight | Control | 16 | 7 | 44 | 19 | 7 | 37 |
| | Treated | 26 | 8 | 31 | 19 | 8 | 42 |
| Moderate | Control | 70 | 44 | 63 | 57 | 26 | 45 |
| | Treated | 63 | 37 | 59 | 65 | 16 | 25 |
| Marked | Control | 7 | 6 | 86 | 3 | 3 | 100 |
| | Treated | 7 | 4 | 59 | 4 | 1 | 25 |
| Severe | Control | 5 | 5 | 100 | 11 | 11 | 100 |
| | Treated | 2 | 2 | 100 | 4 | 3 | 75 |

jection sites are shown in table 2. No significant change was observed in the size of the induration among 16 patients whose control injections resulted in slight reactions. On the other hand, definite reduction in the size of the indurations as compared to those of the controls was noted in those with moderate, marked, or severe reactions. The number of partially inhibited reactions increased along with the increasing severity of the response. Thus, 17 per cent of the moderate reactions were partially inhibited; 43 per cent of those with marked control reactions had moderate reactions at the treated site; 60 per cent of those with necrotic reactions on the control side had no necrosis on the treated side.

Seventy-two hours after the tuberculin injection, the majority of patients with slight reactions on the control side, as well as some of those with negative reactions, had mild aggravation of the reaction following the application of hydrocortisone acetate ointment for the second time. On the other hand, the ointment clearly caused partial inhibition of the moderate, marked, and severe reactions: 16 per cent of those with moderate reactions had lesser induration at the treated site; one of the 3 patients with marked reaction had a slight reaction on the treated side; 7 of the 11 patients (64 per cent) with necrotic control reactions had no necrosis on the treated forearm (table 3).

The influence of topical hydrocortisone acetate ointment on the incidence of side effects of tuberculin hypersensitivity is shown in table 4. Twenty-four hours after the first application of the ointment, it was evident that the hydrocortisone acetate ointment had not significantly altered these side effects. On the other hand, a definite decrease was noted after the repeated application of hydrocortisone acetate ointment on the second day after the test. Among the patients manifesting moderate reactions, there were about one-third as many with itching and painfulness at the treated site as there were with complaints on the control side. Among those with marked reactions, there were about one-fourth as many with side effects at the treated site as there were on the control one.

* * *

This randomized study with adequate controls was undertaken in order to investigate a method of preventing unnecessarily severe reactions and minimizing undesirable side effects due to tuberculin hypersensitivity. Whereas more elaborate precautions could have been taken to eliminate any bias in reading the results, the methods used provided consistent and reproducible measurement of induration at the injection sites by different physicians, who were not informed about the particulars of the study.

The results of the present investigation supported the conclusions of the preliminary investigation. In the majority of patients with necrotic reactions on the control side, necrosis evidently was prevented at the treated site; some of the marked reactions were partially inhibited by the treatment with hydrocortisone ointment; and, in a significant number of instances, the side effects of tuberculin hypersensitivity were alleviated by the treatment.

Three additional observations were made: hydrocortisone acetate ointment did not aggravate a negative reaction; on no occasion was a positive reaction totally inhibited; and alleviation of itching and pain required repeated application of the ointment.

In order to prevent marked and necrotic reactions, hydrocortisone acetate ointment apparently should be applied to each positive reaction site twenty-four hours after the test, inasmuch as side effects cannot be predicted from observation of the twenty-four-hour reactions (table 1).

*In summary:* Investigation of the influence of topical hydrocortisone acetate ointment on the extent of induration, prevention of necrosis, and alleviation of undesirable side effects of positive intracutaneous tuberculin reactions to 5 TU of PPD has confirmed preliminary observations that this procedure may be a valuable adjunct to tuberculin mass surveys.

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May 8, 1959

# A CLINICAL EVALUATION OF THIOCARBANIDIN AND ISONIAZID IN THE TREATMENT OF PULMONARY TUBERCULOSIS

Thiocarbanidin has been studied extensively *in vitro* and in laboratory animals.[1–4] These experiments indicated that the drug possessed definite antituberculous activity, and it was suggested that it might prove to be an adequate substitute for para-aminosalicylic acid. Clinical administration has thus far been limited, and in the one published report no bacteriologic studies were included.[5] The purpose of this paper is to present pertinent clinical and bacteriologic data encountered during the treatment of 41 patients with acute symptomatic tuberculosis, employing combined thiocarbanidin therapy.

All patients with suspected pulmonary tuberculosis who were admitted to the hospital during a specified period of time were considered as candidates for the study. However, only those patients who were tuberculin positive, had had no previous antituberculous therapy, and in whom the diagnosis of active pulmonary tuberculosis was established are included in the report. Background data therefore were constant and patients were unselected.

Bacteriologic studies, including drug susceptibility, were made initially and at monthly intervals. The tests of drug susceptibility were determined by the indirect method prepared from a representative growth on the Löwenstein-Jensen medium using the serial tube method with control.

Resistance to isoniazid was considered to be present when growth of 2 plus or more occurred in tubes containing isoniazid concentrations of 1 $\gamma$ per ml. Resistance to streptomycin and para-aminosalicylic acid were determined in the same manner, and were considered to be present when growth of 2 plus or more occurred in concentrations of 10 $\gamma$ per ml.

Roentgenograms were obtained at intervals of no longer than one month in addition to the usual hemograms, urinalyses, and tests of liver and renal function. Evaluation of the chest films was categorized as showing worsening, no change, or slight, moderate, or marked improvement. Initially 73 patients were accepted in the study and started on therapy. This report includes only those patients in whom therapy was continued for two months or more. Thirty-two patients were excluded because they were either transferred to other institutions or received premature discharges before two months of treatment. Of the 41 patients included in the study, 21 were males and 20 females, of whom all but 4 were Negroes. The ages ranged from thirteen to seventy-seven years. All patients had pulmonary tuberculosis and 4 had extrapulmonary involvement in addition. Two patients had miliary tuberculosis, one with meningitis, one without. All but 2 had either moderately advanced or far advanced disease. No cases of pleural effusion were included.

Thiocarbanidin was administered to all patients in a dosage of 2.0 gm. daily given in four divided doses and at four-hour intervals between 9:00 a.m. and 9:00 p.m. Thirty-nine of the 41 patients received 100 mg. of isoniazid three times a day; one patient, 1 gm. of dihydrostreptomycin pantothenate twice weekly; and the other, 2 gm. of isonicotinyl salicylidene hydrazide (Salizid®) daily in combination with the thiocarbanidin. (Since previous studies[6] indicated that Salizid is therapeutically as effective as isoniazid, the results obtained in the one patient receiving Salizid are analyzed with the isoniazid group.)

*Toxicity:* The drug was well accepted by all patients, and no instances of toxicity or intolerance of any type were encountered.

*Clinical response:* Clinical evaluation was assessed in 40 of the group on the basis of the usual criteria of defervescence, decrease in cough, return of appetite, and weight gain (table 1). Twenty-five patients were considered to have favorable response, and sputum "conversion" occurred in 20. In 2 of the 5 patients in whom reversal of infectiousness did not occur, organisms resistant to isoniazid appeared. Seven patients had a "fair" response, none of whom attained a reversal of infectiousness. A poor clinical response was apparent in 4 patients, and either no response was noted or definite worsening was encountered in the 4 remaining patients. In both of these latter groups, bacilli resistant to the companion drug

[1] YOUMANS, G. P., *et al.*: Am. Rev. Tuberc., 1958, 77, 301.

[2] YOUMANS, G. P., *et al.*: Tr. Seventeenth Conference on Chemotherapy of Tuberculosis, Veterans Administration-Armed Forces, 1958, p. 365.

[3] STEENKEN, W., JR., *et al.*: Tr. Seventeenth Conference on Chemotherapy of Tuberculosis, Veterans Administration-Armed Forces, 1958, p. 368.

[4] DILL, W. A., AND GLAZKO, A.: Tr. Seventeenth Conference on Chemotherapy of Tuberculosis, Veterans Administration-Armed Forces, 1958, p. 373.

[5] PHILLIPS, G.: Tr. Seventeenth Conference on Chemotherapy of Tuberculosis, Veterans Administration-Armed Forces, 1958, p. 380.

[6] KATZ, S., *et al.*: Tr. Thirteenth Conference on Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, 1954, p. 374.

continued to be excreted, and a change of treatment became imperative. In only one patient of the entire group, a young female with minimal disease, was clinical evaluation impossible.

*Roentgenographic course:* In general, results were poor and inferior to those encountered with isoniazid-PAS or other standard regimens or even with isoniazid alone (table 2). Only 7 patients had roentgenographic improvement of a moderate to marked degree. Twelve others showed slight to moderate improvement, while changes of a minimal nature or no improvement were encountered in 18 (44 per cent). Four of the group had definite worsening, manifested in 3 by the occurrence of spread of disease in the contralateral lung, while the fourth developed tuberculous empyema in the fourth month of therapy.

*Bacteriologic results:* Contrary to the clinical course, bacteriologic results were discouraging. Twenty patients, just less than half of the entire group, were able to achieve reversal of infectiousness. Of the twenty-one patients in whom bacteriologic "conversion" did not occur, fifteen (71 per cent) excreted tubercle bacilli resistant to the companion drug. This conversion is compared to the duration of treatment in figure 1, and in table 3 bacteriologic conversions and the development of drug resistance are correlated with the presence of cavities and their subsequent behavior. All of the 6 patients who continued to excrete tubercle bacilli which remained drug susceptible received treatment for more than four months.

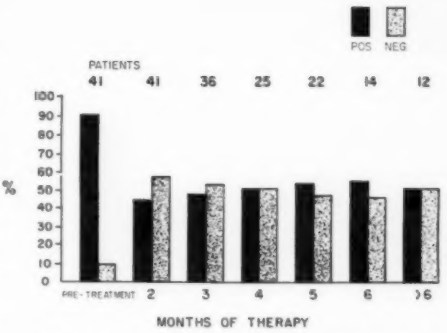


FIG. 1. Comparison of sputum conversion and duration of therapy.

TABLE 3

RESULTS: ACCORDING TO EXTENT OF DISEASE

| | |
|---|---|
| Number of patients without cavities | 9 |
| Sputum conversion | 7 |
| Number of patients with cavities | 32 |
| Sputum conversion | 13 |
| Cavity closure (tomogram) | 9 |
| Drug resistance | 15 |
| With cavitation | 13 |
| Without cavitation | 2 |

TABLE 1

RESULTS: CLINICAL RESPONSE

| Type of Response | Number of Patients | Per Cent | Bacteriologic Status | | Drug Resistance |
|---|---|---|---|---|---|
| | | | Negative | Positive | |
| Favorable | 25 | 61 | 20 | 5 | 2 |
| Fair | 7 | 17 | 0 | 7 | 5 |
| Poor | 4 | 10 | 0 | 4 | 4 |
| None or worsening | 4 | 10 | 0 | 4 | 4 |
| Unable to evaluate | 1 | 2 | 0 | 1 | 0 |

TABLE 2

RESULTS: ROENTGENOGRAPHIC CHANGE

| Degree of Change | Number of Patients | Per Cent |
|---|---|---|
| Marked to moderate | 7 | 17 |
| Moderate to slight | 12 | 29 |
| Slight to none | 18 | 44 |
| Worsening | 4 | 10 |
| Totals | 41 | 100 |

Eleven of the group of 15 patients in whom drug-resistant organisms were encountered had pretreatment cultures which were positive for tubercle bacilli; and, in all, the strains were drug susceptible (table 4). One patient's pretreatment culture was negative for tubercle bacilli and the culture remained negative throughout the first four months of therapy. During this time, both clinical and roentgenographic improvement was readily apparent. In the fifth month, return of fever was noted, a repeat chest film revealed a new area of infiltration in the contralateral lung, and the sputum culture obtained at this time was positive for tubercle bacilli and the organisms were resistant to isoniazid.

Three of this group of patients did not have available pretreatment cultures due to a mechanical failure of the incubator. In 2 patients, however, cultures negative for tubercle bacilli were obtained in the second month of therapy, and both patients subsequently developed clinical and roentgenographic relapse. Tubercle bacilli were cultured at this point and the organisms were

TABLE 4
DRUG SUSCEPTIBILITY

| Months of Treatment | Number of Patients | Cultures | | | Drug Susceptibility | | |
|---|---|---|---|---|---|---|---|
| | | Positive | Negative | Unknown | Susceptible | Resistant | Unknown |
| Initial | 15 | 11 | 1 | 3 | 10 | 0 | 1 |
| First | 15 | 6 | 1 | 8 | 2 | 1 | 3 |
| Second | 15 | 8 | 3 | 4 | 2 | 6 | 0 |
| Third | 15 | 10 | 3 | 2 | 0 | 8 | 2 |
| Fourth | 11 | 7 | 2 | 2 | 1 | 6 | 0 |
| Fifth | 10 | 7 | 2 | 1 | 2 | 5 | 0 |
| Sixth | 7 | 5 | 1 | 1 | 0 | 5 | 0 |
| Sixth to twelfth | 5 | 4 | 1 | 0 | 1 | 3 | 0 |

isoniazid resistant. No initial improvement was noted in the other patient. In the third month of therapy tubercle bacilli were cultured for the first time. The organisms were resistant to both isoniazid and streptomycin although he had not received the latter drug. It is possible in this case that the organisms may have been drug resistant prior to the institution of therapy.

* * *

The data available from observation of these 41 patients indicate that thiocarbanidin in the dosage employed is not an effective antituberculous agent under clinical conditions. Original *in vitro* and *in vivo* studies suggested that it had no great potential when used alone, but seemingly may have been an adequate substitute for PAS, which would mean that it might be a satisfactory "companion drug" for either isoniazid or streptomycin. The ideal companion drug should have as its primary functions the ability to prevent the emergence of drug resistance to the major antituberculous agent and to increase the incidence of bacteriologic "conversion." In studies conducted previously by the Veterans Administration and at this institution,[7, 8] patients with essentially the same type of disease treated with isoniazid alone had a "conversion" rate of about 50 per cent. Furthermore, a comparison of different "double-drug" regimens, i.e., isoniazid-PAS, or streptomycin-PAS, both when the streptomycin was administered daily or twice weekly, have a conversion rate substantially more than 50 per cent.[9, 10] Previous studies from this institution utilizing dihydrostreptomycin, Salizid, and hinconstarch[11, 12] also have indicated higher conversion rates, less drug resistance, and a significantly higher incidence of clinical and roentgenographic improvement.

In this study, a little less than 50 per cent of the patients had sputum negative for tubercle bacilli at the end of three months. More important, however, is the fact that, of the 21 patients in whom sputum "conversion" did not occur, resistance to isoniazid (or streptomycin in one case) developed in 15 (71 per cent). Further confirmation of the ineffectiveness of thiocarbanidin is available by an analysis of the clinical and roentgenographic response of this group. Seven of the total of 41 patients had evidence of moderate to marked roentgenographic clearing; slight improvement was noted in 12; either no improvement or slight clearing occurred in 18 patients, while definite worsening was apparent in the remaining 4 patients.

The experience encountered in this study suggests that isoniazid when administered alone is as efficacious or even slightly superior to the combination of thiocarbanidin and isoniazid. If this is true, some explanation is necessary to substantiate this discrepancy. Synergistic action has been previously reported by Steenken and associates[3] when the two drugs were employed in *in vitro* studies and, therefore, any antagonism seems unlikely. Another obvious cause could be the lack of absorption of thiocarbanidin from the gastrointestinal tract. Previously Dill and Glazko[4] have demonstrated low but adequate concentrations of the drug in the blood. Six patients in this study had drug concentrations in the blood which were theoretically adequate, although low. Both

[7] KATZ, S., *et al.*: Tr. Twelfth Conference on Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, 1953, p. 90.

[8] DYE, W.: Tr. Twelfth Conference on Chemotherapy of Tuberculosis, Veterans Administration, Army, and Navy, 1953, p. 111.

[9] LIVINGS, D.: Tr. Seventeenth Conference on Chemotherapy of Tuberculosis, Veterans Administration-Armed Forces, 1958, p. 19.

[10] MUSCHENHEIM, C., *et al.*: Tr. Seventeenth Conference on Chemotherapy of Tuberculosis, Veterans Administration-Armed Forces, 1958, p. 117.

[11] DONOHOE, R. F., *et al.*: An evaluation of hinconstarch in the treatment of acute tuberculosis (unpublished data).

[12] DONOHOE, R. F., *et al.*: Tr. Seventeenth Conference on Chemotherapy of Tuberculosis, Veterans Administration-Armed Forces, 1958, p. 349.

of these possibilities should be studied further before thiocarbanidin is discarded as an antituberculous agent.

*In summary:* Thiocarbanidin and isoniazid or streptomycin were administered to 41 patients with acute pulmonary tuberculosis. Roentgenographic clearing was marked in 7 patients and moderate in 12. In 18 patients, either slight or no improvement was noted, and roentgenographic worsening was observed in 4 patients. The over-all clinical response was not striking, and bacteriologic "conversion" occurred in slightly less than 50 per cent. Furthermore, in 15 (71 per cent) of the 21 patients who failed to achieve sputum "conversion," the patient strains of tubercle bacilli demonstrated resistance to the companion drug. As a result of this study, thiocarbanidin in its present form would appear to be an ineffective antituberculous agent when employed in a dosage of 2.0 gm. daily.

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March 11, 1959

[12] Portions of this investigation were conducted during the tenure of a Residency Fellowship sponsored by the American Trudeau Society, medical section of the National Tuberculosis Association.

# *Books*

BACTERIAL AND MYCOTIC INFECTIONS OF MAN. Edited by René J. Dubos. Pp. 820, J. B. Lippincott Company, Philadelphia, 1958. Price, $8.50.

The new edition of this well-known textbook keeps the general outline of preceding editions, although the text has largely been rewritten. The subject matter is arranged to illustrate the underlying theme, the host-parasite relationship. To begin with, there are general chapters describing, on the one hand, the morphology, physiology, and pathogenic ability of the parasite and, on the other, the immunologic reactions produced by it in the host. This section is rounded off and placed in perspective in a new chapter by Dr. Dubos on the evolution and ecology of microbial diseases. There follow systematic accounts of the various bacterial genera and the diseases they cause in man arranged so as to illustrate different types of parasitic relationships. Finally, the last section is concerned with applied bacteriology. The normal flora of the human body is described and then there are chapters on sterilization and chemotherapy, epidemiology, and diagnostic bacteriology.

The general standard of presentation is very high, each chapter being a concise and accurate account of a subject which brings the reader to the borderline of advancing knowledge with remarkable ease and clarity. Even more pleasant to report, it remains really interesting, a rare feature in textbooks. In part this is because the authors have managed to communicate their enthusiasm for their subjects, and in part because they have not been afraid to include material and views that are modern and controversial. This is a book that bridges the gap between medicine and bacteriology and would be of particular value to the physician with an interest in bacteriology or to a bacteriologist who wishes to refresh his knowledge in unfamiliar fields. There is perhaps rather too little detail in the systematic accounts, and the references are too few to help the working bacteriologist in his own speciality.

A new chapter on the chemotherapy of microbial diseases by Dr. Walsh McDermott is of particular interest to readers of the AMERICAN REVIEW OF RESPIRATORY DISEASES. An attempt has been made to show how chemotherapeutic activity is modified by the host-parasite relationship, and how little we really know from the highly artificial *in vitro* systems commonly used. This is a difficult subject to write about because of the complexity of the systems involved and the lack of precise evidence about many of them. Dr. McDermott has written an account which is in most respects excellent, but surely he has gone a little astray when he argues that the advantages of multiple drug therapy are due principally to more effective action on the susceptible bacterial population and not to the prevention of resistance. Many of his examples are drawn from the field of tuberculosis, and it is unfortunately true that the emergence of resistance is a major catastrophe for the tuberculous patient and one that in the end often leads to his death.

D. A. MITCHISON
London, England

THE YEAR BOOK OF GENERAL SURGERY—1958-1959 SERIES. Edited by Michael E. DeBakey. Pp. 588 (149 figures), The Year Book Publishers, Inc., Chicago, 1958. Price, $7.50.

Some of the more significant articles from the surgical literature of the past year are abstracted and digested in this small volume. Although the majority of items selected are from American journals, foreign contributions of value have been included.

All phases of surgery, from basic physiology to details of operative procedures, are covered. In view of the recent advances in the field of cardiovascular, aorta, and peripheral artery surgery, it is not surprising that this subject occupies 128 of the 588 pages. However, this is not to imply that the other branches of surgery are neglected. Recent experimental and clinical experience in problems of the head and neck, breast, thorax and mediastinum, lungs and pleura, peripheral veins, liver and spleen, biliary tract, pancreas, esophagus, stomach and duodenum, small intestine, appendix, colon and rectum, hernia, and the genitourinary tract are also reviewed. Throughout the book, the papers have been abstracted in a clear, concise way; pertinent illustrations and tables are included, and experimental methods or operative techniques are summarized briefly but adequately enough to be followed without difficulty.

A valuable addition to the text is the comment following articles dealing with subjects with which

the editor has had personal experience. Whether he refutes or substantiates the results of a paper, his comment influences the reader. It appeared to this reviewer that the comments were made not in the spirit of passing judgement, but in the analytic, critical, introspective manner of one who has attempted a similar line of endeavor and has reached a conclusion as to its worth.

The last section of the book is devoted to contributions in the field of anesthesia, covering such topics as depressant drugs, ventilation, circulation, inhalation anesthesia, relaxants, and regional and spinal anesthesia. In these chapters, too, the work is abstracted in clear, simple fashion.

It is impossible to review a book of this type in detail, since it covers such a wide variety of topics. In general, however, it may be said that this digest of current surgical experiences, both experimental and clinical, is extremely well done. It is difficult to prepare a comprehensive, thorough précis of a scientific article, but the editor has artfully accomplished the task. Certainly an abstract, no matter how well done, is never as good as the original article, and for those desiring more information, full references to the original articles are included following each summary. This is a valuable source book of current thought for investigators, clinicians, and students at all levels.

S. Frank Redo
New York, New York

BCG Vaccination Against Tuberculosis. By Sol Roy Rosenthal. Pp. 389, Little, Brown and Company, Boston, 1957. Price, $7.50.

Dr. Rosenthal has collected and summarized in this book the huge literature on BCG vaccination from its beginnings in 1909 through 1956. He first sketches the basic facts on immunity in tuberculosis and what is known of its mechanisms. He then reviews the literature on the use of vaccines other than BCG against tuberculosis. A first-hand report on the early history of his work with Calmette on their vaccine is given by Dr. Guérin of Paris.

The chapters on the cultural characteristics of BCG, the host response, and the methods used to prepare, standardize, and preserve the vaccine will be of interest primarily to the specialists in the field. Of more general interest are the chapters on the routes and methods of administration of the vaccine. Dr. Weill-Hallé of Paris, summarizes his pioneering experiences with the oral vaccination procedure. Dr. H. Wallgren of Stockholm reports on the development of the intradermal vaccination technique. The author reviews the transcutaneous vaccination method which he initially introduced and which has tended to become the preferred method.

The problems involved in the tuberculin testing before and after vaccination are adequately discussed, and the literature on the experimental study of BCG vaccination in various animal species is reviewed. Of the most interest to the reviewer is the description of the large controlled studies of BCG vaccination in man which have been made in Scandinavian countries, Great Britain, and America, and the evaluation of their results. A chapter dealing with the incidence of untoward reactions following BCG vaccination points out that with the multiple puncture method these complications are almost completely avoided. Finally, the role of BCG vaccination within a comprehensive tuberculosis control program is defined.

This book will be useful to those who wish to become readily acquainted with the literature on BCG, or who wish to be informed about specific problems concerned with BCG vaccination procedures.

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Biología del Cancer. By José Abelló. Pp. 142, Libréria Cientifico Médica Española, Madrid, 1958.

This monograph by a well-known worker in the field of tuberculosis and diseases of the chest has special significance for readers of The American Review of Respiratory Diseases in its imaginative approach, through the present maze of conflicting opinion, to the etiology of cancer and, particularly, carcinoma of the lung. Abelló believes he has evidence for the production of an "autovirus" from the cells of the host which can become a cell parasite and, specifically, for the production, in a very few cases, of experimental cancer of the lung following injection of the sputum of treated pulmonary cancer patients. An abundance of pertinent literature is cited, although without the desirable full journal references.

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Pedlar Mills, Virginia

## BOOKS RECEIVED

TUBERKULOSE-JAHRBUCH 1957. Edited by Rolf Griesbach. Pp. 291, Springer-Verlag, Berlin, Gottingen, Heidelberg, 1959.

CORONARY HEART DISEASE. ANGINA PECTORIS; MYOCARDIAL INFARCTION. By Milton Plotz. Pp. 353, Hoeber-Harper, New York, 1957. Price, $12.00

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# *American Trudeau Society*

**Medical Section of the National Tuberculosis Association**

## NOTICES

The following dates and locations of the next five annual meetings of the National Tuberculosis Association and the American Trudeau Society have been selected and approved:

1960—Los Angeles, California—May 15–20
1961—Cincinnati, Ohio—May 21–26
1962—Miami Beach, Florida—May 20–25
1963—Denver, Colorado—May 12–17
1964—New York, New York—May 24–29

### FOURTH NATIONAL CANCER CONFERENCE

The Fourth National Cancer Conference, sponsored by the American Cancer Society, Inc., and the National Cancer Institute, will be held in Minneapolis, Minnesota, on September 13–15, 1960. The theme of the Conference will be: "Changing Concepts Concerning Cancer."

For further information, write: Medical Affairs Department, American Cancer Society, 521 West 57th Street, New York 19, New York.

### POSTGRADUATE COURSE ON PULMONARY FUNCTION

For the tenth successive year, a postgraduate course on "The Measurement of Pulmonary Function in Health and Disease" will be given at the Boston City Hospital. The dates are March 21–25, 1960; tuition is $75.00.

Sponsored by the Medical Schools of Harvard University, Tufts University, and Boston University; the Harvard School of Public Health; the Massachusetts Tuberculosis and Health League; and the Massachusetts Trudeau Society, the course is aimed at physicians who are interested in diseases of the chest and wish to acquaint themselves with the methods used in the evaluation of pulmonary function. The methods used in the evaluation of pulmonary function and related cardiac function will be demonstrated.

The deadline for applications is March 1, 1960. Applications and detailed information may be obtained from: Edward J. Welch, M.D., 1101 Beacon Street, Brookline 46, Massachusetts.